

**THE DEVELOPMENT AND HATCHING
OF THE EGGS OF
ASPICULURIS TETRAPTERA (NEMATODA: OXYURIDA)**

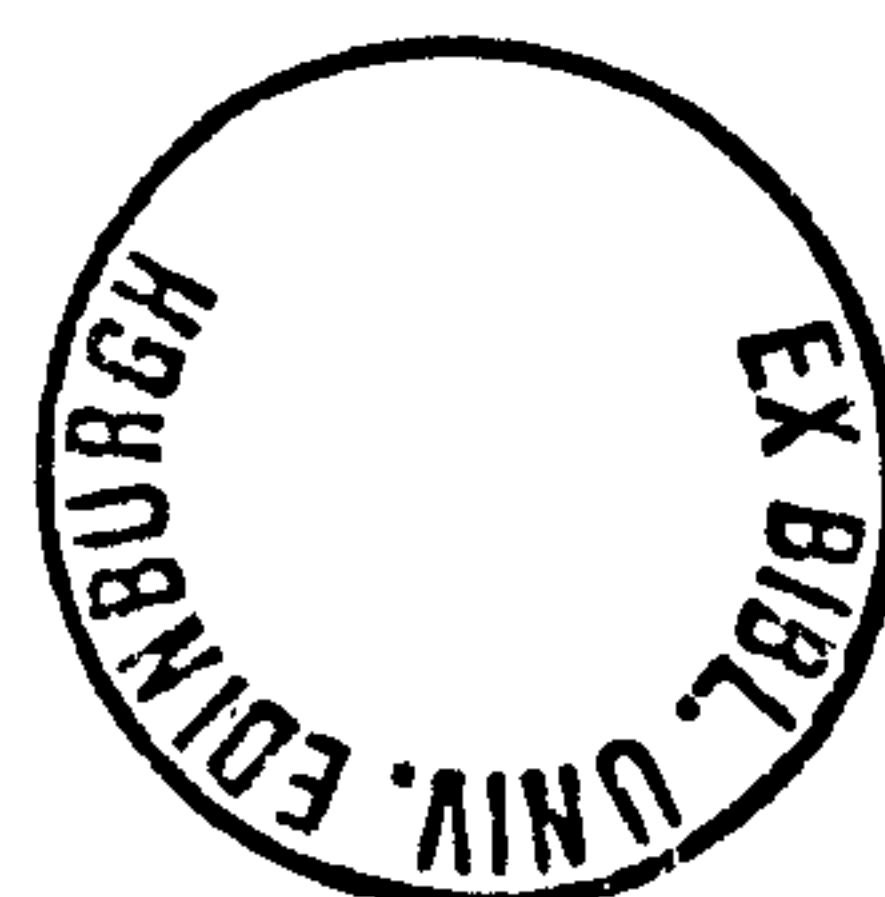
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I hereby declare that this thesis was composed by myself and the work it contains is my own.

Abstract

In the oxyurid *A.tetraptera*, egg development, hatching *in vitro* and infectivity *in vivo* was reinvestigated following difficulties encountered in obtaining adequate hatch using a method devised by Anya (1966). The population egg development of faecal and uterine eggs was examined at a range of temperatures (15–37°C). In comparison with Anya's (1966a) study using uterine eggs, the rate of larval development within both faecal and uterine eggs and the level of larval disintegration increased with increasing temperature. In contrast, the rate of egg development was increased at equivalent temperatures and spontaneous hatching occurred. Spontaneous hatching increased with temperature. More faecal eggs developed than uterine eggs in the present study, reasons are suggested for this observation. The level of development of uterine eggs in the two studies was similar. Hatching *in vitro* and infectivity *in vivo* occurred after a shorter period of incubation than reported by Anya (1966a & b). Uterine eggs and faecal eggs were compared at the individual egg level. No difference has been found in egg dimensions, rate of larval shrinkage in concentrated NaCl solution and TEM and SEM ultrastructural studies. Faecal eggs offered a slight increase in protection to the embryo from ultra-violet light. Uterine eggs were found to be at an earlier stage of development than faecal eggs when collected. Explanations are offered for the differences between the results of Anya's studies (1966a & b) and the present study.

Physico-chemical factors were examined for their stimulatory effect on hatching *in vitro* following an inability to obtain an equivalent level and rate of hatch, using a method, found by Anya (1966) to give optimum hatching. He obtained 80% hatch after incubating eggs in 0.1M phosphate buffer (pH 7.3–7.4), at 37°C, for 7 hours, in contrast to less than 25% hatch obtained in the present study after 20 hours. Levels of hatch increased in a selection of "Good" buffers (pH 6–10). Maximum number of fully emerged larvae (over 80%) occurred in 0.05M Bicine buffer (pH8.5), at 37°C, after 20 hours. Rates of hatch increased when eggs were incubated sequentially in a combination of treatments including, pepsin-HCl, trypsin and dithiothreitol, at 37°C, reaching maximum levels of hatch (over 80%) after approx.6 hours incubation. Although initial embryonation was found to occur in NaCl solutions of varying

concentrations, increasing incubation in concentrated solutions caused larval shrinkage and inhibited hatching when eggs were stimulated in 0.05M buffer at 37°C. Hatching was inhibited when eggs were stimulated to hatch in osmotic concentrations over approx. 130mOsm/Kg. Increasing osmotic concentration decreased the rate and level of emergence obtained. An explanation is offered for the osmotic effect on hatching. Eggs were found to initiate hatching in the stomach. Fully emerged larvae were recovered from the lower small intestine and the caecum 30– 60 minutes post infection. Hatching *in vivo* was increased in contrast to results obtained by Anya (1966b). An explanation is offered for the difference in rates of hatching *in vivo*

Using the rate of larval shrinkage in concentrated NaCl solution, the pattern of staining in OsO₄, TEM and SEM ultrastructural studies, the same sequence of permeability changes was found to occur gradually in ageing eggs and rapidly in eggs stimulated to hatch by pepsin-HCl, trypsin, dithiothreitol and buffer, at 37°C. Pepsin and trypsin only stimulated hatching at 37°C. Dithiothreitol and HCl also stimulated hatching at 21°C. Evidence provided by OsO₄ staining and SEM studies, indicate that these stimuli directly affect the eggshell. Temperature (37°C) stimulated larval emergence. The observation that eggs hatched at 21°C, after brief stimulation and increased larval activity with temperature suggested that larvae were actively involved in hatching. An enzyme assay for proteolytic activity in eggs during hatching produced inconclusive results because of bacterial/fungicidal contamination. Larval behaviour was examined during hatching using time lapse analysis. Explanations are offered for the sequence of permeability changes which occurred in the eggshell during ageing and after stimulation to hatch and, the role of the larva in the mechanism of hatching. A literature review has been completed on the structure, development, survival and hatching of nematode eggs in general.

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TABLE OF CONTENTS

1 GENERAL INTRODUCTION	1
2 GENERAL MATERIALS AND METHODS	2
2.1 TECHNIQUES EMPLOYED	2
2.1.1 The maintenance of <i>A.tetraptera</i> in laboratory mice	2
2.1.2 Extraction Techniques	3
2.1.3 The storage and incubation of <i>A.tetraptera</i> eggs	4
2.1.4 The estimation of percentage hatch	4
2.1.5 The preparation of <i>A.tetraptera</i> eggs for electron microscopy	5
2.1.6 Time lapse video recording techniques	7
3 EGG DEVELOPMENT	8
3.1 LITERATURE REVIEW	8
3.2 POPULATION EGG DEVELOPMENT	20
3.2.1 Introduction	20
3.2.2 Materials and methods	25
3.2.3 Results	27
3.2.4 Discussion	31
3.3 INDIVIDUAL EGG DEVELOPMENT	36
3.3.1 Introduction	36
3.3.2 Materials and methods	39
3.3.3 Results	41
3.3.4 Discussion	46
4 HATCHING	50
4.1 LITERATURE REVIEW	50
4.2 HATCHING <i>in vitro</i> AND <i>in vivo</i>	67
4.2.1 Introduction	67
4.2.2 Procedures and Results	73
4.2.3 Discussion	92
4.3 PHYSIOLOGY OF HATCHING	99
4.3.1 Introduction	99
4.3.2 Experimental procedure and results	101
4.3.3 Discussion	114
5 DISCUSSION	121
6 BIBLIOGRAPHY	131

CHAPTER 1

GENERAL INTRODUCTION

Initially the aim of this thesis was to make a comparative study of the physiology of hatching of several oxyurid parasites, from both homoiothermic and poikilothermic hosts. However interesting observations made during attempts to stimulate *A.tetraptera* Schulz (Oxyuroidea;Nematoda) to hatch using a method described by Anya (1966) led to a detailed study of the development and hatching of this one nematode. In Chapter 3, a comparative study of population development and infectivity made with Anya's study (1966a) produced some new information concerning spontaneous hatching. Differences between uterine and faecal eggs were also investigated at the individual egglevel, using timelapse video analysis and ultrastructural observations. The literature concerning the development and survival of eggs is reviewed.

Hatching *in vivo* and the effects of various physico-chemical factors were investigated in Chapter 4, in an attempt to increase the rate and level of hatch of *A.tetraptera* eggs. The changes which occurred in the egg when stimulated to hatch and during development prior to spontaneous hatching were examined using various techniques including susceptibility to osmotic stress, staining in osmium tetroxide and ultrastructural studies. Literature concerning the mouse intestinal environment, hatching stimuli and hatching mechanisms of nematodes is also discussed

The significance of the findings on egg development and hatching of *A.tetraptera* is discussed in relation to those found for other nematodes in Chapter 5.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Techniques Employed

2.1.1 The maintenance of *A.tetraptera* in laboratory mice

In order to provide a constant supply of *A.tetraptera* eggs it was necessary to maintain infections of the worm in laboratory mice.

As natural infections of *A.tetraptera* are frequently found in many laboratory mouse colonies (Hoag, 1961; Stone and Maxwell, 1966) an initial survey was undertaken to assess whether this applied to the assorted mouse populations housed in the departmental animal house. Many of the strains of mice available were infected, in particular the CBA and CBA/Ca/T6 strains.

The prevalence of *A.tetraptera* in laboratory mice suggests that the parasite is ideally suited to the animal house environment. According to Anya (1965), eggs are infective seven days after their release thus allowing for reinfection in mice when cages are cleaned less often than a six day interval. As the eggs are borne on air currents (Westcott, Makzewski and van Hoosier, 1976) cross infections can occur between cages housed in enclosed spaces.

Mice were housed in standard plastic cages on sawdust litter in numbers not exceeding 12 mice per cage and were cleaned out weekly. Pellet food (Diet 41B Oxoid) and water were supplied *ad libitum*. Normally the routine care of mice was adequate to ensure continuing infections.

Stahl (1961) and Benke (1975) have shown that the age and sex of infected mice affect the uniformity and size of the worm burden. In general mature males harbour larger and more sustained infections than other mice (Mathies, 1954; Behnke, 1975), therefore mature male CBA and CBA/Ca/T6 mice were used to maintain infections of *A.tetraptera*.

The maintenance of experimental infections was also achieved by the oral passage of infective eggs. However, this method was found to be time consuming and potentially distressing to the mice and was discontinued in

favour of the natural infections already present in many mice.

2.1.2 Extraction Techniques

The extraction of *A.tetraptera* eggs from faecal matter

To collect faecal matter mice were placed in a clean cage without litter overnight, water and food were given *ad libitum*. Eggs were extracted from collected faeces using a salt flotation technique modified from Lane (1923) and described below.

The faeces were softened with a little water and ground to a smooth paste using a mortar and pestle. The sample was washed through a nylon mesh teastrainer to remove coarse debris and the strained fluid centrifuged in a flat topped test tube (Paine Scientific Apparatus Ltd) until the formation of a solid faecal pellet. The supernatant was discarded and the solid pellet agitated with a small amount of saturated NaCl, to resuspend the sediment. The tube was almost filled with saturated NaCl, inverted several times to distribute the sediment evenly, replaced in the centrifuge and filled with saturated NaCl to form a positive meniscus. A 22 by 22mm coverslip (Chance Proper Ltd) was carefully placed on the surface of the tube to form an airless seal and the tube centrifuged for about two minutes at a low speed. The coverslip was removed with a deliberate movement and the suspended eggs liberally washed off with distilled water into a 9cm plastic petri dish (Sterilin Ltd) marked on its base with an approximately 2cm square grid, using a scalpel blade. The faecal eggs were collected from the debris using a flame pulled fine ended pipette, stored in a plastic petri dish (grid marked) containing 0.05M phosphate buffered saline (P.B.S.), pH 7.2 (Oxoid Ltd) and incubated as described in Section 2.1.3. unless otherwise mentioned.

The extraction of eggs from gravid females of *A.tetraptera*

Anyia (1966) collected large numbers of eggs laid by gravid females maintained in physiological saline at 37°C for 2-3 hours. Despite several attempts at this method, using various physiological salines and incubation times, the incubated gravid females only released a small part of their egg burden. Therefore to obtain uterine eggs gravid females were mashed through

a nylon meshed teastrainer into a grid marked 9cm petri dish containing P.B.S. Uterine eggs were collected from the debris as described in Section 2.1.1.

2.1.3 The storage and incubation of *A.tetraptera* eggs

From a study investigating the effect of temperature on embryonic development (Section 3.2.), it was found that storing unembryonated eggs for several weeks at 4°C had no adverse effect on their eventual development and viability.

For the majority of experiments freshly collected eggs were available, however stored eggs were used in experiments requiring large numbers of eggs, where used their period of storage is stated.

When preliminary experiments were conducted concerning the culture of eggs to the infective stage, conflicting results were produced in comparison to those obtained by Anya (1966). Therefore a detailed study was carried out investigating the effect of temperature on the development of infectivity as described in Section 3.2. From the results obtained it was found that optimal development and subsequent hatching was achieved when eggs were incubated at 21°C for 5-15 days in daily changes of P.B.S. (20 ml) containing a working concentration of penicillin/streptomycin solution (Sigma Ltd) in grid marked petri dishes. This method of incubation was used to obtain infective eggs as required throughout the study unless otherwise mentioned.

2.1.4 The estimation of percentage hatch

Initially it was necessary to establish a standard assay for larval hatching. From preliminary experiments it was apparent that two visible stages of hatching could be observed, larvae protruding through the operculum (emerging larvae) and free larvae in the incubating medium. Thus to calculate hatching a count of the number of larvae at each stage was made at periodic intervals throughout an experiment. This method of data collection allowed the stages of hatching to be analysed either separately or combined as appropriate.

To estimate percentage hatch either of two methods were used, depending on the number of eggs being sampled and the duration of the experiment. In

both cases, the total number of eggs was taken as the number of eggs capable of hatching, i.e., those which had developed (contained vermiform larvae, Section 3.2.).

In short term experiments where replicates of small numbers of eggs (25-100) were observed over a period of hours, the original number of eggs in each replicate was counted plus the number of emerging and free larvae at the end of the experiment, thus allowing the percentage hatch to be calculated.

In long term experiments where large numbers of eggs (1000's) were observed over a period of several days, samples of 50-100 eggs were taken from the total population at intervals and the percentage hatch in each of these samples was then determined.

2.1.5 The preparation of *A.tetraptera* eggs for electron microscopy

In order to shed further light on various stages of development and hatching, eggs were examined using electron microscopy. Due to the problems of penetrating the lipid layer of nematode eggs with fixatives and embedding resins (Bird, 1971), several fixation techniques were tried on *A.tetraptera* eggs. The most successful procedure, described below, was adapted from a method used by Wharton (1979).

Transmission electron microscopy

Eggs were either recovered from gravid female worms or mice faecal matter. The eggs were extracted from both sources of material as described in Section 2.1.2. The eggs were fixed in Karnosky's fixative (Pearse 1960) for two hours, washed in 0.1M sodium cacodylate buffer (pH 7.2), (Sigma Ltd.), several changes, overnight, post-fixed in 1% osmium tetroxide (Sigma Ltd.) in cacodylate buffer for 45-60 minutes, rinsed twice in cacodylate buffer, and once in water (15 minutes each) prior to dehydration through a series of acetone concentrations (15 minutes each) until placed in 100% acetone (3 rinses, 15 minutes each) and left in acetone/Epon mixture (Emscope Ltd.), (3-1 ratio) overnight. The worms were embedded by passing through a series of acetone/Epon mixtures of increasing resin strength, changed

every 24 hours, finishing in 100% medium hard Epon resin. The resin was polymerized for 16 hours at 60/70°C.

This method of preparation was used for all specimens examined, however the procedure was modified on occasions where the form of treatment given to the eggs prior to fixation required squashing of the eggs to allow the entry of fixative and/or extended fixation times. Where this was necessary it is mentioned in the text.

Thin sections (60–80nm) were cut using glass knives on a Reichert OMU4 ultramicrotome, picked up on formvar/carbon-coated copper grids and stained with uranyl acetate (Sigma Ltd.), (2% aqueous) for 1.5 hours followed by Reynold's lead citrate solution (Pearse, 1960) for 5 minutes. Sections were examined using a Jeol 100S Transmission electron microscope, operated at 80kV.

Low Temperature Scanning Electron Microscopy

Eggs were prepared using the Emscope SP2000 cryogenic preparation system (Emscope Laboratories Ltd, Kingsnorth Ind.Est., Ashford, Kent, TN23 2LW), (Beckett and Read, 1986). Specimens to be examined were attached to a dedicated copper cryo stub using a thin layer of Tissue tek cryo adhesive (Lab- Tek division, Miles Laboratories Inc, Naperville, Illinois 60540). Cryo fixation was carried out by plunging the stub into melting Nitrogen (-210°C) under an atmosphere of dry argon. The stub was shrouded and then transferred under vacuum to the specimen stage of the scanning electron microscope for initial examination and freeze etching of surface contamination. This was accomplished by raising the microscope specimen stage temperature from -160°C to -65°C. The specimen was then transferred to the main chamber of the SP2000 at -186°C for sputter coating with gold in an argon atmosphere at a pressure of 0.08 Torr for 3 minutes with a deposition current of 20mA. Final observation and photography was carried out with the specimens at -160°C in a Cambridge Stereoscan 250 scanning electron microscope, fitted with a "top hat" anode using beam accelerating voltages of 5–10KV.

Light microscopy

Thin sections (1µm) fixed and embedded as above were stained with 1% toluidine blue in 1% borax for about 3 minutes and were examined using a Vickers microscope.

2.1.6 Time lapse video recording techniques

The technique described below for investigating the development and hatching of eggs was designed by Matthews (1985). A group of 15-20 eggs were transferred by micro-pipette to the central pillar of a slide tissue culture chamber (Cruickshank *et al*, 1959), (Sterilin Ltd.). Excess incubation fluid was removed and the eggs were orientated with a mounted bristle and covered with a coverslip sealed to the chamber surface with silicone grease. A thermistor was introduced into the annular cavity of the chamber and the whole preparation transferred to the warm stage of a microscope fitted with a video camera and connected through a video timer to a National NV8030 time-lapse video tape recorder. The entire chamber was covered with an insulated brass bridge, fitted with a second thermometer to conduct heat around the chamber. The temperatures were monitored with a Grant recording thermometer. The eggs were continuously recorded, normally in 80 hours mode, this is equivalent to one frame being recorded every 1.44 seconds and it provided adequate resolution for general behavioural parameters to be observed. For some experiments the automatic alarm facility of the recorder was used which allowed 3 minutes of recording at "normal speed" (one frame every 0.02sec.) providing greater resolution for analysing larval activity in detail. The interval between such recordings was normally set at one hour.

CHAPTER 3

EGG DEVELOPMENT

3.1 Literature Review

A.tetraptera is a member of the order Oxyuroidea and inhabits the upper colon of its mouse host. It appears to have no obvious debilitating effect on its host, although heavy infections may result in rectal prolapse due to rectal irritation (Hoag, 1960). The life cycle of *A.tetraptera* is simple and direct, eggs voided with the host faeces develop first stage larvae which obtain maximum infectivity after one week's incubation in water at 24°C (Anya, 1966a). Following ingestion by the host, the eggs hatch and the larvae which emerge in the lower intestine migrate into the crypts of Lieberkuhn (Anya, 1966b; Behnke, 1974). After a period of 4-5 days, the worms return to the lumen of the colon and on day 7 migrate anteriorly to its proximal region where they mature into adults (Behnke, 1974). Viable eggs are released by gravid females, 24 days after infection (Anya, 1966b). Although no evidence of moulting was observed in the egg or in the host (Philpot, 1924 and Anya, 1966b), Philpot considered some shedding of the cuticle occurred as the male worm lost the ventral cuticular expansion of the tail 14-18 days after infection. Measurements made of various aged larvae suggested that development was a gradual process of growth. Anya (1966b) deduced from growth curves that three moults occurred in the life cycle of *A.tetraptera* after ingestion by the host. He suspected that the first moult occurred prior to migration into the crypts of Lieberkuhn, the second while in the crypts and the final moult to the adult worm between the 15th and 18th day in the lumen of the colon.

Studies of the egg development of *A.tetraptera* outside the mouse host (Philpot, 1924; Deschiens, 1944; Hsu, 1951; Chan, 1953; Anya, 1965, 1966a and Wharton, 1980) and larval development within the mouse host (Philpot, 1924; Chan, 1955, Anya, 1966b and Mya, 1955, Fisher, 1958 cited Behnke, 1974) suggest that it has a life cycle similar to other oxyurids examined.

It has been suggested that the oxyurid type of life cycle is the simplest among nematodes parasitic in vertebrates (reviewed by Chandler *et al*, 1941; Otto, 1966) and follows the typical nematode pattern of 6 stages, an egg, four

juveniles and an adult. However a literature search of the various oxyurid life cycles studied (Schwartz, 1923; Philpot, 1924; Dikmans, 1931; Wetzel, 1931; Dobrovolny & Ackert, 1934; Cram *et al*, 1943; Todd, 1944; Chitwood & Chitwood, 1950; Prince, 1950; Chan, 1952; Stahl, 1963; Otto, 1966). has failed to give a complete account of the number of moults present in the life cycles of any of the oxyurids studied. The presence of a second stage larva within the egg has been reported for three species of oxyurid, *Enterobius vermicularis* (Chitwood in Chandler *et al*, 1941), *Leidynema appendiculata* (Dobrovolny *et al*, 1934) and *Hammerschmidtella diesingi* (Todd, 1944).

Nematode egg embryonation has been reviewed by Nigon (1965), Hope (1974) and more recently Ehrenstein & Scherenberg (1980). The process of embryonation includes such characteristic features as determinate cleavage, chromatin diminution and cell constancy. Although nematodes share the same pattern of development, the time interval and conditions required for the completion of embryonation may vary considerably among different species (Anya, 1976; Croll & Matthews, 1977). In general, nematodes which hatch to give active free living larvae tend to spend less time in the egg than nematodes which rely on ingestion of embryonated eggs for transmission or plant parasitic species (Crofton 1961, Thorne 1961 and Wallace 1963).

In the Trichostrongylidae (Crofton 1961), species of the genera *Oesophagostomum*, *Chabertia*, *Bunostomum*, *Trichostrongylus*, *Haemonchus* and *Cooperia* form first stage larvae which emerge from the egg after only 24-48 hours at 20-25°C and subsequently form infective third stage larvae. Whereas in species of the genus *Nematodirus*, the development of all three larval stages occurs within the egg, the infective larvae emerge after 18-30 days at 20-28°C. In *Trichuris ovis* the egg containing the first stage larvae is the infective stage. The egg is infective after a period of development of 20-23 days at 28°C.

In the Oxyuroidea the degree of development at deposition shows considerable variation. Eggs of *A.tetraptera*, *H.diesingi* and *L.appendiculata* are at the multicellular stage when passed in the faecal matter (Anya, 1965 and Hominick *et al*, 1974), whilst eggs of *E.vermicularis*, *Oxyuris equi*, *S.muris* and *S.obvelata* contain tadpole stage larvae when laid on the host's perianal skin (Schwartz, 1923; Philpot, 1924; Cram, 1943 *et al*; Chan, 1952 and Stahl, 1961,

1963). In general eggs released in host faeces are in early segmentation stages whereas eggs laid on the perianal region of the host are embryonated.

Environmental factors which affect the development and survival of the free living stages of nematodes include temperature, availability of oxygen, humidity and osmotic forces. The extent of influence of the surrounding microclimate is dependent on whether the transmission stages are incorporated within faecal material or soil, its consistency and rate of breakdown. Extensive research examining the influence of these conditions on the free living stages has been completed under controlled laboratory and field conditions and are reviewed by Chandler & Read (1961), Thorne (1961), Wallace (1961, 1963), Rogers (1962), Rogers & Sommerville (1963) and Evans (1974). Observations indicate that conditions necessary for development include i) an optimum temperature in the range of 20–30°C, with a minimum temperature of 10–15°C, ii) oxygen and iii) an adequate moisture supply.

The requirements for the embryonation of oxyurid eggs appear to be in agreement with those of nematodes in general. Eggs of *A.tetraptera*, *E.vermicularis*, *S.obvelata*, *H.diesingi* and *L.appendiculata* failed to develop in deaerated water (Philpot, 1924, Dobrovolsky & Ackert, 1934; Todd, 1944; Anya, 1965, 1966a and Wharton, 1980). No embryonation was observed in *A.tetraptera* eggs incubated in water, initially boiled and gassed with nitrogen (Anya, 1966a and Wharton, 1980). According to Zawadowsky & Schalimov (1929b), *E.vermicularis* eggs could only develop to the tadpole stage inside the host intestine and required oxygen to reach the infective stage although they were not killed by short exposure to oxygen-free conditions. Dobrovolsky & Ackert (1934) observed that *L.appendiculata* eggs in O₂ water mounts under coverslips showed arrested development but continued to develop when additional air was admitted. Todd (1944) found that embryos in *H.diesingi* eggs failed to moult in a partial vacuum and an atmosphere containing 75% carbon dioxide & 75% hydrogen gas. An increase in these gases in the atmosphere to 100% CO₂ & 100% H₂ was found to be lethal, destroying any embryos present.

Studies available suggest that oxyurids can develop at temperatures between 15 and 37°C and the rate of development increases with temperature. *A.tetraptera* eggs were found to take between 65 hours at 20°C and 12 hours at 30°C to obtain optimum development (Philpot, 1924; Deschiens, 1944; Hsu,

1951; Chan, 1953; Anya, 1965, 1966a and Wharton, 1980). This literature is reviewed in full in Section 3.2.1. In *E.vermicularis* eggs, coiled moving embryos (ring and a half stage) were found 30-40 hours after deposition (Philpot, 1924; Jones & Jacob, 1941 and Deschiens, 1944). The infective ring and a half stage of *S.obvelata* was reached in 20-24 hours at 29°C (Chan, 1952). *H.diesingi* eggs contained active embryos after 36-48 hours at 26°C, the resting infective stage was reached in an additional 36 hours (Todd, 1944). However Wharton (1980) observed that the infective stage was reached after 22-31 hours at 26-34°C.

At 37°C, moving embryos were found in *A.tetraptera* eggs within 24 hours by Philpot (1924) and Hsu (1951) and in 2-3 days by Anya (1966a), however the larvae rapidly became moribund at this temperature. Deleterious effects have not been reported for oxyurids which are deposited around the perianal region, at this temperature. In eggs of *E.vermicularis*, *S.obvelata* and *S.muris*, development is rapid, the infective ring and a half stage is formed in 6 hours, 5 hours and 4 hours respectively at 37°C (Jones and Jacob, 1941; Chan, 1952 and Stahl, 1963). *L.appendiculata* eggs were found to embryonate satisfactorily at 37°C forming the active stage after 12 hours and the resting infective stage in a further 3 to 7 days (Dobrovolny & Ackert, 1934).

Moisture also plays an important part in the development of oxyurid eggs. The eggs of oxyurids deposited in host faecal matter develop when submerged in water, whereas those eggs which are laid around the perianal region of the host though requiring high humidities for optimum development, deteriorate under such conditions (Philpot, 1924, Deschiens, 1944 and Chan, 1952). *A.tetraptera* eggs developed in a variety of media such as distilled water, saline, horse serum and saliva (Philpot, 1924 and Hsu, 1951). Wharton (1980) found the optimum humidity for the development of *A.tetraptera* and *H.diesingi* eggs was 98% relative humidity and the eggs also developed in 30-50% ethanol. Dobrovolny & Ackert (1934) cultured *L.appendiculata* eggs on slides in 1/3N Ringers solution, stacked in moist chambers to prevent drying. Philpot (1924) found that the shell of *E.vermicularis* could not withstand prolonged exposure to water. Optimum development occurred under varying conditions of humidity, between 47 and 91% depending on the temperature (Jones and Jacob, 1941; Deschiens, 1944). *S.obvelata* eggs failed to develop in tap water, distilled water, Ringer's solution or dilute formalin solution. Chan (1952) found

incubation in very moist conditions opened up the operculum of the eggs prematurely. Optimum development occurred when eggs were kept in a dry watch glass contained in a humid chamber. The transmission stage of many parasites, egg and larval, can withstand low temperatures for considerable periods of time (reviewed by Hyman, 1951, Thorne, 1961, van Gundy, 1965, Evans, 1974). In some parasitic species, for example *Nematodirus battus* (Christie, 1962; Parkin, 1972), *Heterodera avenae* (Fushtey & Johnson, 1966; Banyer & Fisher, 1971b) and *Meloidogyne naasi* (Siddiqui & Taylor, 1970; Franklin *et al*, 1971), the infective larvae pass the winter in the egg and even require a period of exposure to reduced temperatures for optimum hatching to occur in the following spring. The necessity of winter chilling of the eggs of these three species prior to their hatching at higher temperatures ensures that the eggs avoid hatching in the summer or the autumn. They only hatch in the spring when conditions are favourable for the growth of plant in the case of *M.naasi* and *H.avenae* and when new born lambs are susceptible to infection by *N.battus*. Similarly, arrested development may also be shown by larval stages of several trichostrongyles in the host. Delayed development is shown by fourth stage larvae of *Haemonchus contortus* in sheep (Blitz & Gibbs, 1971) and *Ostertagia ostertagi* in cattle (Armour, 1970) and is thought to be induced by the effect of climatic factors on third stage larvae on pasture in autumn. The arrested larvae mature in the spring resulting in an increase in the faecal egg population and thus a greater likelihood of infection in recently born animals. Michel (1974) has reviewed the literature on arrested development of animal-parasitic nematodes.

The survival of oxyurid eggs at low temperatures has been investigated in two species (Jones & Jacob, 1941, Chan, 1953). *A.tetraptera* eggs at all stages of development survived storage at temperatures of 4°C for extended periods (Chan, 1953). Temperatures of -8 to -12°C were fatal in a few days to eggs of *E.vermicularis*, but "excellent survival" was noted after 18 days at 3-5°C (Jones and Jacobs, 1941).

The infective stage of many animal and plant parasitic stages survive long periods of desiccation as reviewed by Hyman (1951), Wallace (1961), Evans (1974) and Evans & Perry (1976). In particular, tylenchids are notoriously resistant to desiccation, second stage juveniles of *Anguina tritici* survived in dry wheat galls for 9 years, dry cysts of *Heterodera* remained viable for years

although the number of healthy eggs decreased steadily (Franklin, 1938) and "eelworm" wool, that is aggregates of fourth stage larvae of *Ditylenchus dipsaci* have been shown to survive at least 23 years (Fielding, 1951). Ascarid eggs are highly resistant to desiccation, at temperatures of -5 to -10°C, eggs removed from dried female pig ascarids developed naturally after 2 years. At room temperature survival is limited to 2 years and under wet, cool conditions, 4 to 5 years (Martin, 1926; cited Hyman, 1951). The degree of resistance shown by the eggs and infective larvae of trichostrongylids varies among the species (Furman, 1944; Prasad, 1959; Rose, 1961; Anderson & Levine, 1968; Ellenby, 1968a; Waller & Donald, 1970a; Parkin, 1976 and Wharton, 1982). Recent research among the trichostrongylids and the tylenchids has shown how different stages of the same species vary in their ability to resist desiccation. In particular the increased survival of embryonated eggs and infective third stage larvae to desiccation as compared to the unembryonated eggs and first and second stage larvae (Anderson & Levine, 1968; Waller & Donald, 1970a; Perry, 1977a, 1977b and Wharton, 1982). This marked resistance to desiccation has been attributed largely to a decrease in the rate of drying of the eggs or larvae due to the ability of the enclosing cyst, egg, sheath and/or cuticle to provide an effective barrier against water loss (Ellenby, 1968a,b; Waller, 1971 and Parkin, 1972). The eggshell of *Globodera rostochiensis* and the retained 2nd stage cuticle of *H. contortus* have both been found to decrease in permeability as they dry (Ellenby, 1968a,b). Waller (1971) suggested that the increased resistance of *T. colubriformis* to desiccation when compared to *H. contortus* (Waller & Donald, 1970) was due structural differences in the eggshell. Parkin (1972) reported a change in eggshell structure from morula to third larval stage which may be connected with the resistance to desiccation shown by the unhatched third stage larva.

The formation of aggregations enhances the survival of a number of plant parasitic nematodes (Evans & Perry, 1976). Ellenby (1968b) has suggested that as the outer layer of the clump dries, it forms a barrier enhancing the survival of the worms in the centre by reducing the rate of water loss. Clumping has also been found to increase survival in infective *T. colubriformis* larvae when exposed to extreme desiccation (Wharton, 1982). Coil formation has been shown to reduce desiccation in plant and animal parasitic nematodes (Evans & Perry, 1976) and more recently *T. colubriformis* larvae (Wharton, 1982).

Oxyurid eggs of various species, in common with a number of other nematode eggs appear to be remarkably resistant to desiccation. Viable eggs of *A.tetraptera* and *E.vermicularis* were found in laboratory and household dust (Nolan & Reardon, 1939 and Hoag, 1960). Dobrovolny & Ackert (1934) found that unembryonated eggs of *L.appendiculata* were less resistant to desiccation than embryonated eggs. Philpot (1924) and Chan (1953) found excessive moisture or dryness rendered eggs of *S.obvelata* inviable. Jacobs and Jones (1941) completed studies on the survival of *E.vermicularis* eggs under known conditions of humidity and temperature. Results indicated a detrimental effect on pinworm eggs from lowering the humidity and/or raising the temperature. Cool, dry conditions (20-24.5°C & relative humidity, R.H.30-54%) and warm, dry conditions (27-29°C & R.H.38-50%) were unfavourable for survival. No eggs survived after one minute at 65°C and R.H. of 20%. Wharton (1980) examined the effect of desiccation on oxyurid eggs, *H.diesingi* and *A.tetraptera*. He found the rate of water loss from the eggs as indicated by their collapse was dependent upon the relative humidity and temperature of the environment. The eggs of *H.diesingi* had a slower rate of water loss than *A.tetraptera* eggs. At 76% relative humidity, 20°C, 50% collapse occurred within 12 days for *H.diesingi* and 4.5 days for *A.tetraptera* eggs. Thus the oxyurid eggshell slows down but does not prevent loss of water from the egg.

The formation, chemistry, structure and function of nematode eggshells have been examined by several workers. This work has been extensively reviewed by Christenson (1950), Fairbairn (1957), Rogers (1962), Bird (1971), Anya (1976) and Wharton (1980a). The eggshell structure varies considerably between different orders of nematodes and even different species. The nematode eggshell may consist of a variety of layers. The oxyurid eggshell is an example of the most frequently observed pattern, that is 3 endogenous layers consisting of an inner lipid layer, a middle chitinous layer and an outer vitelline layer and 1 or 2 uterine layers secreted exogenously by the uterine cells, as reviewed by Wharton (1980).

In general oxyurid eggs are small, but some species, for example *S.obvelata* are over 100 microns long (Christenson, 1950). Studies completed on the oxyurid eggshell are described below. Histological and histochemical studies have been performed on the eggs of *A.tetraptera* (Anya, 1964; Wharton, 1979b), *E.vermicularis* (Jones & Jacobs, 1939; Hulinska & Hulinsky, 1973, cited

Wharton, 1980), *H.diesingi* (Wharton, 1979c) and *S.obvelata* (Wharton, 1979d). The abnormalities found in the descriptions of the structural property of *A.tetraptera* eggs are due to the limitations of magnification at the light microscope level. Philpot (1924) reported the presence of an operculum and radial striations on the surface of several oxyurid eggs, including *A.tetraptera*. Monne and Honnig (1954) further investigated the eggshell properties of *O.equi*, *E.vermicularis*, *A.tetraptera* and *P.ambigurus*. They considered the eggwall to consist of two shells, an outer shell of quinnone tanned protein and an inner chitinous shell with a lipid layer attached to its inner surface. Anya (1964) considered *A.tetraptera* eggs to consist of 3 layers, an outer lipoprotein layer, a middle chitinous layer and an inner lipoidal- glycosidal membrane. From an ultrastructural and histochemical study, Wharton (1979b) found a further two layers in the *A.tetraptera* eggshell. He found an inner lipid layer, a middle chitinous layer and an outer vitelline layer formed endogenously by the egg and two exogenous layers, the internal and external uterine layers secreted by the uterine cells. He deduced that Anya (1964) had misidentified the layers of the eggshell. Instead he concluded that the outer lipoprotein layer was the external uterine layer, the middle chitinous layer was the internal uterine layer and the inner lipoidal - glycosidal membrane was the chitinous layer. From his studies on the oxyurid eggshell, Wharton (1979a,b,c & d) found the basic eggshell structure of members of the Oxyuroidea to be similar in the endogenously produced layers with differences occurring in the internal and external uterine layers. In *H.diesingi* and *S.obvelata* the spaces of the internal uterine layer are discrete and do not form an interconnecting system unlike those in *A.tetraptera* which open to the exterior via pores in the external uterine layer. A characteristic of oxyurid eggshells is the operculum, the area through which the larva emerges on hatching. Operculation shows considerable variation among the Oxyuridae examined, as reviewed by Christenson (1950). Wharton (1979b,c,d) found the operculum consisted of a modification of the uterine and chitinous layers. In *A.tetraptera* eggs the whole area of the operculum is modified, the internal uterine layer is thinner and an increase in the frequency of breaks occurs in the external uterine layer. The chitinous^{layer} contains a fibrous electron dense region beneath the vitelline layer. In *S.obvelata*, the same modifications occur in the uterine layers of the operculum but only at the opercular groove. A thickening of the chitin layer occurs which also contains an electron dense region.

The function of the nematode eggshell including that of oxyurids, is reviewed by Wharton (1980, 1980a). He described the different layers of the egg and the variation in eggshell structure of different nematode orders and species, in terms of their ability to protect the enclosed embryo from the external environment. Previous opinions have suggested that the resistance of the nematode eggshell to environmental hazards was due to its impermeable nature (Arthur & Sanborn, 1969; Barrett, 1976; Fairbairn, 1957, 1961; Rogers & Sommerville, 1963). However embryonic development of nematodes has been shown to be aerobic in many species of nematode (Passey & Fairbairn, 1955; Nikandrow & Blake, 1972), including several oxyurid species, (Philpot, 1924; Zawadowsky & Schalimov, 1929b; Todd, 1944; Dobrovolsky & Ackert, 1954; Anya, 1966a and Wharton, 1980). The infective larvae of oxyurids are enclosed within the egg for their entire period of development outside the host, therefore the eggshell must be permeable to oxygen sized molecules. The permeability of the eggshell to oxygen demonstrates that the egg is also permeable to smaller sized water molecules (Wharton, 1980). Evidence of water permeability in various nematode species has been demonstrated by Ellenby (1968), Wharton (1979) and Clarke & Perry (1980) in *G.rostochiensis*, *Ditylenchus dipsaci* and *A.suum* eggs which have been shown to lose water following desiccation and osmotic stress. Matthews (1985) found increasingly hypertonic solutions directly affected the behaviour and development of *A.tubaeforme* and *A.ceylanicum* larvae within the egg. By measuring the water content of artificially and naturally hatched larvae in solutions which allowed larvae to hatch normally, Matthews (1986) showed there was no difference in the water contents of the two types of larvae, suggesting that the eggshell provided little resistance to the passage of water. In the oxyurids, *A.tetraptera* and *H.diesingi*, Wharton (1980) has shown the eggs lose water at a slow rate dependent upon the relative humidity and temperature. From calculations made on diffusion, Wharton (1980) has found that if gaseous exchange is restricted to the pores of the uterine layer, the area available for water loss is much reduced. Thus the function of the uterine layers may be to allow an adequate supply of oxygen for embryonic development, whilst preventing desiccation of the egg.

Studies on the permeability of the nematode eggshell by investigating the rate of water loss from and the penetration of fixatives and dyes (Wharton, 1979e, 1980; Barrett, 1976; Bird & McClure, 1976) indicate that the main

permeability barrier is the lipid layer in ascarids, tylenchids and oxyurids. This barrier is destroyed after exposure to high temperatures (60-65°C). The time of exposure and whether the eggs are allowed to cool before exposure to desiccation, dyes and fixatives was found to be an important factor in determining the temperature at which the barrier was destroyed. Their data suggests that there is not a single "critical" or "transition" temperature, but a gradual melting point of the components forming the lipid layer.

The chitinous layer is often the thickest layer of the nematode eggshell and provides structural strength (Wharton, 1980). Chitin has been detected by X-ray crystallography in eggs of *A.lumbricoides* and by infra red spectroscopy in *T.suis* eggs (Wharton & Jenkins, 1978). In oxyurids, the chitin layer is the thickest layer of the eggshell, however Wharton (1979a,b,c & d) found no histochemical evidence of its presence in oxyurids. It may be present in too small quantities, or masked by protein, as protein is frequently found in association with chitin. Anya (1964) inferred its presence from the histochemistry of the detection of glucosamine in acid hydrolysates.

The function of the vitelline layer in nematodes is uncertain. Matthew's (1986) suggested that it may be associated with selective permeability.

Eggs collected from host faeces are brown and insoluble in many reagents whereas uterine eggs are transparent and soluble in acids, alkalis and various enzymes. From studies on the chemical composition of a variety of nematode eggs including *A.tetraptera*, Monne (1954b, 1955, 1959, 1962) has suggested that quinone tanning of various layers of the eggshell as the egg passes through the host gut, may be responsible for the change in colouration and solubility of the egg. Anya (1964b) could not demonstrate the presence of phenols and phenolases in either the reproductive system of the female *A.tetraptera* or the eggshell. Wharton (1979a) suggests that evidence for quinone tanning in *A.tetraptera* is inconclusive.

It has been suggested that the brown colouration of the eggshell protects the embryo from the harmful effect of the sun's rays. The effect of radiation on eggs of several species of nematode has been examined (Wright & MacAllister, 1934; Duggar, 1936). Its effect on the viability of oxyurid eggs has been observed in two species, *L.appendiculata* (Dobrovolsky & Ackert, 1934) and *E.vermicularis* (Shalimar, 1935; Hollaender *et al*, 1940 and Jones *et al*,

1940). Dobrovolsky & Ackert (1934) found that development ceased in *L.appendiculata* eggs exposed to strong indirect sunlight or the rays of a 400 watt projection lamp passed through water for 15 minutes; continued exposure killed the embryos. Shalimar (1935) observed a detrimental effect on eggs not yet developed to the infective stage, using a commercial mercury vapour lamp (wavelength unspecified). Hollaender *et al* (1940) examined the effect of known wavelengths of monochromatic radiation in the ultra-violet region between 2200A & 2950A. Maximum susceptibility was found at 2805A. The tadpole stage of development was most vulnerable to the ultra- violet radiation tested. Jones and Jacobs (1941) using the ability of fully developed embryos to hatch found ultra-violet radiation had a delayed lethal effect causing a decreased rate of hatch and impaired viability of hatched larvae.

The ability of oxyurid eggs to survive the environmental conditions to which they are exposed is essential for transmission of the infective larvae to the definitive host. From the studies described below it is apparent that some types of oxyurid eggs are capable of several modes of infection, which may enhance their chances of ingestion by the host. The four most likely routes of infection are i) dissemination of eggs in air currents and household dust, ii) ingestion of eggs from contaminated material in the environment, iii) grooming behaviour and iv) retrofection.

Wescott, Makyewski and Hoover (1976), have shown that eggs of *A.tetraptera* can reach uninfected mice kept separately from infected mice but in the same room, suggesting dissemination by air currents. Nolan and Reardon (1939) reported the presence of *E.vermicularis* eggs in household dust. Transmission of eggs can also occur in humans via grooming behaviour (finger transmission) and indirect contamination of objects and food material in the environment (Cram *et al*, 1943).

Schuffner and Swellengrebel (1949), demonstrated the occurrence of retrofection in *E.vermicularis* infections. Retrofection occurs in oxyurids when larvae hatched out of eggs laid on the perianal region of the host migrate up the rectum to re-establish infection in the large intestine. Hsu (1951) showed that retrofection was a possibility in *A.tetraptera* infections. However the experiments he performed were of an artificial nature. He incubated eggs at 25°C for 6 days before placing them on the anal region of mice restrained in

movement. At post-mortem, larvae were found migrating up the colon. It is probable that retrofection would not be likely in natural infections. Anya (1966, 1966a & b), found eggs required laboratory incubation before infection. Prince (1950) recovered recently hatched living *S.obvelata* larvae from the posterior portion of the large intestine of the rat host, however Chan (1952) failed to obtain retrofection when eggs were placed on the perianal region of the host. He found eggs required 8 hours incubation at 37°C before infective larvae were obtained in the laboratory. He therefore maintained that constant licking of the perianal region by the host would not allow for this period of incubation and unregulated bowel movements also ruled out retrofection.

Under natural conditions it has been suggested that transmission of oxyurid eggs of *H.diesingi* and *L.appendiculata* would normally occur from cockroaches constantly feeding on infected food or faecal material (Dobrovolsky & Ackert, 1934; Todd, 1944). However Hominick & Davey (1974) found two instances of precocious development, whereby females of the pinworm *L.appendiculata* were found to harbour eggs at the infective stage of development. They suggested that it was possible that i) the adult females of *L.appendiculata* were of an advanced age and the rate of passage of the eggs out of the female was slowed, thus resulting in precocious development., ii) perhaps the environment of the nematode changes in weakened hosts, resulting in early development of their eggs, which would be advantageous to the species as the weakened host would be eaten by other members of the cockroach colony thus acquiring infection and iii) precocious development would be beneficial for autoinfection as a result of the grooming behaviour.

3.2 Population Egg Development

3.2.1 Introduction

Factors affecting the development of A.tetraptera eggs outside the host

In this chapter some of the factors which affect the development of *A.tetraptera* eggs outside the host have been re-examined following difficulties encountered in obtaining adequate levels of hatching *in vitro* using the method described by Anya (1966). In these preliminary experiments eggs were incubated in distilled water at 24°C for 8-10 days, the period of development required prior to obtaining maximum hatch on application of the hatching stimulus (Anya, 1966a). Eggs were then stimulated to hatch by incubation in a 0.1M phosphate buffer, pH 7.5, at 37°C for 8 hours (Anya, 1966). Despite replication of the above experiments percentage hatches of only 15 and 25% were obtained. It was also observed that these levels of hatch, though low, were achieved after shorter periods of development than suggested by Anya (1966a).

In view of the relevance of Anya's work (Anya 1966, 1966a) to the continuation of further studies on the hatching of *A.tetraptera* in this thesis, detailed studies were made on some aspects of development to resolve the conflicts obtained in the preliminary experiments.

The experiments described in this chapter deal with the effect of a range of temperatures on population egg development examined in terms of the number of larvae which develop, emerge spontaneously during their period of incubation, hatch *in vitro* when stimulated and are capable of infection *in vivo*. The effect of various osmotic concentrations on egg development and subsequent hatching *in vitro* was also examined to determine whether the use of distilled water as an incubation medium adversely affects development, since eggs are subjected to different osmotic stresses from those of their natural environment, mouse faeces. Hsu (1951) observed that larval development was apparently normal in saline concentrations of up to 0.85% however he did not investigate the effect of osmotic stress on larval viability.

Where possible the development of both uterine and faecal eggs was

examined in order to determine whether the conflicting results obtained between the preliminary experiments and Anya's data (Anya, 1966a) on the optimum developmental period and *in vitro* hatching were in fact due to previously undetected differences between faecal and uterine eggs. Uterine eggs as opposed to faecal eggs have been used by the majority of authors in previous studies, therefore unless otherwise mentioned it can be assumed that the descriptions below concern uterine eggs (Philpot, 1924; Hsu, 1951; Chan, 1953; Anya, 1966a and Wharton, 1980).

Literature review

Several authors, Philpot (1924), Deschiens (1944), Hsu (1951), Chan (1953), Wharton (1980) and especially Anya (1965, 1966a) have studied egg development. To evaluate the effect of environmental factors on larval development in the egg, various parameters have been used including i) the development of vermiform larvae, ii) the onset of larval motility, iii) hatching *in vitro* and iv) infectivity *in vivo*.

Philpot (1924), Hsu (1951) and Anya (1966a) have observed that an increase in developmental temperature resulted in a decrease in development time and the number of larvae which finally developed. Philpot (1924) observed the presence of motile larvae in eggs incubated in water after 65–68 hours at 22°C and 20 hours at 37°C. At 37°C a smaller percentage of eggs developed and, if maintained at this temperature the eggs began to die after 2 or 3 days. Hsu (1951) incubated eggs in normal horse serum at 25°C, after 17 hours the eggs contained small vermiform larvae, after 41 hours the embryos had increased in length and after 65 hours incubation they were moving in their shells. He also reported a deleterious effect on egg development at 37°C with 4 in 5 larvae moving after 24 hours incubation in both horse serum and water at 28°C and only 1 in 8 larvae moving under the same conditions at 37°C. Motile larvae were also observed by Deschiens (1944), in faecal eggs after 40 hours incubation in tap or distilled water and by Chan (1953) after 2–4 days incubation in distilled water at 27°C.

Anya (1965) reported the development of first stage larvae in faecal eggs after 3 days at 20–30°C. Unlike previous authors who observed the effect of temperature solely in terms of the development of discernible larvae, Anya (1966a) investigated its physiological effect on the ability of larvae to hatch *in*

vitro and *in vivo*. He observed that the rate of egg development was much faster at higher temperatures with maximum levels of development being reached after 3 days, 5-6 days and 9-10 days at 37 °C, 30°C and 24°C respectively, however maximum levels of development were reduced at higher temperatures (see fig.1c-e). He also found that at high temperatures the proportion of fully developed eggs capable of infecting the host was reduced. In brief he concluded that eggs only attained maximum hatching *in vitro* and infectivity *in vivo* after 9-10 days development at 24°C, suggesting that a period of incubation existed in the development of infective eggs during which the infective larvae within the eggs, though morphologically well developed were incapable of hatching *in vitro* or initiating an infection *in vivo*.

According to Anya (1966a) studies made by Philpot (1924), Hsu (1951) and Chan (1955) supported the above conclusions, however a closer examination of their work suggests this may not be the case. Philpot (1924) states that:

"no infection was obtained by feeding mice with eggs containing immature embryos (incubated for one day at 25°C), the eggs were recovered unchanged from the caecum and rectum after 4 hours. When fed with eggs which had been incubated for 6 or 7 days at 25°C, infection followed in most cases."

Anya (1966a) may have assumed that eggs aged between 1 and 5 days old were tested for infectivity.

From the observations on egg development and hatching reported by Hsu (1951) it is difficult to make comparisons of the hatching ability of different aged larvae as often the developmental stage of the eggs used was unclear and in many cases, when specified, the use of various experimental conditions make comparisons invalid.

Chan (1955) described the distribution of *A.tetraptera* larval stages in the intestine of mice. From the method of infection he employed, a single source of stored infective eggs was used to obtain infections, the development of larval infectivity could not be assessed. The age of the eggs used was not specified, however reference to a previous paper by Chan (1953) suggested the eggs were incubated for 5-7 days at 27°C, that is the time required for full development as reported by Wells (1951a), who states:

"this optimum embryonation time confirms a previous report of Philpot (1924)"

Unfortunately no details are given by Wells (1951a) of how he estimated the optimum embryonation time which confirmed the development of infectivity by Philpot (1924). Therefore a reassessment of the development of larval infectivity seemed necessary, firstly because of the various assumptions made by previous authors and secondly the experiment in which Anya (1966a) assessed infectivity precluded a full analysis of the development of infectivity at different temperatures because of the low rate of larval recovery from the mouse intestine. This may have been due to the low initial dose of eggs (100 eggs per mouse) administered.

A further observation of the effect of temperature on egg development was made by Chan (1953) who reported that eggs at all stages of development can survive extended periods of incubation (at least 40 days) at low temperatures of 1-4°C.

The effect of various media on the development of eggs was examined by Philpot (1924) and Hsu (1951). Hsu (1951) found no difference in the development of eggs incubated in water, horse serum and varying saline concentrations (0.25-0.85%). Philpot (1924) observed that eggs developed normally in water and saliva, but in 0.5-0.8% sodium bicarbonate solution larvae initially developed and then died, in normal saline a 20% reduction was observed in development, and only a small percentage developed in 0.2% hydrochloric acid.

Eggs failed to develop in deaerated water which had been boiled and gassed with nitrogen whilst cooling (Anya, 1966a; Wharton, 1980 and personal observations), suggesting that larvae require an external supply of oxygen to complete development. Investigations by Wharton (1980) suggest the uterine layers of the eggshell enable the egg to receive an adequate supply of oxygen, whilst reducing water loss. He found that eggs of *A.tetraptera* have a slow rate of water loss, 50% collapse taking 4.5 days at 76% relative humidity at 20°C which suggests the eggshell slowed down but did not prevent the loss of water from the egg. When exposed to desiccation eggs lost water at a rate dependent upon relative humidity and temperature, however eggs survived and developed normally at 98% relative humidity (Wharton 1980), but did not

develop when incubated in a dry room atmosphere (relative humidity unspecified) at 21–24°C (Anyá 1966a).

3.2.2 Materials and methods

Egg development in vitro

Temperature

Unembryonated *A.tetraptera* eggs were obtained from faecal matter (1-5 day storage at 4°C) and gravid female worms, as described in Section 2.1.2 and, incubated as detailed in Section 2.1.3, at a range of temperatures. In the case of faecal eggs, 5 petri dishes each containing several thousand eggs were incubated at 15°C, 21°C, 25°C, 31°C and 37°C. As fewer uterine eggs were available only 2 petri dishes were incubated, ^{as detailed.} The first at 21°C, equivalent to their natural environmental temperature outside the host, the second at 25°C, the optimum incubation temperature according to Anya (1966).

For each temperature, 22 replicate samples of 30-100 eggs were examined every 24 hours for evidence of development as denoted by the presence or absence of vermiform larvae and evidence of spontaneous emergence i.e. those larvae which at the temperature of incubation hatched without specific stimulation. Spontaneously hatched larvae were removed from all samples prior to artificially stimulating hatching *in vitro*.

As the levels of hatching obtained in the preliminary experiments using Anya's method (1966) were unsatisfactory a detailed study of *in vitro* hatching was undertaken (Section 4.2.). Several techniques were employed each of which reached an equivalent total hatch despite different rates of hatching. The method used in this chapter to assess the ability of developed larvae to hatch *in vitro* involved incubating eggs in 0.05M Bicine buffer (Sigma Ltd), pH 8.5, at 37°C overnight. This procedure whilst not the most efficient in terms of rate of hatching was most similar to that used by Anya (1966), where infective eggs were incubated in a 0.1M veronal acetate buffer, pH7.5, at 37°C for 8 hours. The results are presented in Figs.1 & 2.

Osmotic concentration

To examine the effect of osmotic stress on larval development and viability unembryonated faecal eggs were collected and extracted (Section 2.1.2) and samples each containing 150-250 eggs were incubated at 25°C (Section 2.1.3)

in a range of osmotic concentrations (65–1919mOsmKg) made up from saline solutions (0.03–1M) (Hodgeman, 1983), plus a distilled water (control) for 9–10 days. Two samples were incubated per solution and the number of larvae which developed and hatched *in vitro* expressed as percentages.

To obtain some indication of the osmotic concentration of faecal pellets, samples of ground faecal matter were suspended in visking tubing (0.6mm, Scientific Instruments Centre Ltd) in various NaCl solutions of known osmotic concentration (1,000–10,000mOsm/kg) and examined for any swelling, shrinking, weight loss or weight gain. The results are presented in Fig.3.

Infectivity in vivo

To examine the development of infectivity uterine and faecal eggs were collected (Section 2.1.2) and incubated at 25°C (Section 2.1.3) in small conical flasks. The concentration of the eggs was adjusted to give the required dose (300 eggs in 0.1 ml of suspension). A magnetic stirrer was used to ensure an even egg distribution in the suspension and egg counts on sample doses showed that the number of eggs in subsequent doses varied by no more than 10%. The inoculum was administered orally via a stomach tube every 24 hours to 12 week old infection free CBA male mice (Zoology Department, Edinburgh University), temporarily anaesthetised with ether (M & B Ltd.). Two mice were used per treatment with faecal eggs and one mouse with uterine eggs because of the relative numbers of eggs available. A control consisted of 4 infection free mice housed under the same conditions as the experimental mice.

Weekly egg counts were made on faeces collected overnight. Three egg counts were made per faecal sample and the mean \pm SE count is given in Table 1. In those mice which developed infections eggs were first observed in the faeces after a 25–30 day patent period. Mice which showed no evidence of infection were sacrificed and a thorough search was made of the large intestine for any male or immature worms present.

3.2.3 Results

Temperature

Larval development

Fig.1. compares the percentage larval development in faecal and uterine eggs at different incubation temperatures with the data obtained by Anya (1966a) in an equivalent experiment.

The rate of development of faecal eggs increased with increasing temperature, the maximum number of larvae had developed at 25°C, 31°C and 37°C by the initial observation on Day 1. Larval development was slower at 21°C and 15°C, the maximum number which developed was reached by the observations on Day 2 and Day 5 respectively. The maximum levels of development (80-90%) reached at the above temperatures were initially similar and remained constant at 15°C, 21°C and 25°C, however disintegration of larvae occurred after 10 days incubation at 31°C and 4-7 days incubation at 37°C resulting in a decrease in viable larvae.

The development time of larvae in faecal and uterine eggs was similar at 21°C and 25°C, however 15% less developed in uterine eggs at both temperatures, as compared to faecal eggs.

Maximum egg population development occurred in less than 24 hours for faecal eggs at 25°C, 31°C and 37°C and uterine eggs at 25°C. This differed considerably from the results obtained by Anya (1966a), who found that maximum levels of development were reached in uterine eggs after 9-10 days at 24°C, 5-6 days at 31°C and 3 days at 37°C. His levels of uterine egg development were much reduced when compared with the levels of development for faecal eggs at similar temperatures, however at 24-25°C both sets of uterine eggs attained an equivalent level of development (75%).

Emergence

Fig.2. shows the daily spontaneous emergence of larvae during the incubation of faecal eggs at different temperatures and the effect of the development temperature on their ability to hatch *in vitro* when stimulated at

Fig.1.The effect of temperature on the population development of faecal and uterine eggs.

Faecal eggs (●—●) were examined daily at 15°, 21°, 25°, 31° and 37°C and uterine eggs (○—○) at 21° and 25°C. For each value, n=22 replicates of 30-100 eggs each. Unless depicted standard error lines are covered by the symbols. The results obtained by Anya (1966a) for the development of uterine eggs at 24°, 30° and 37°C are also shown (■—■).

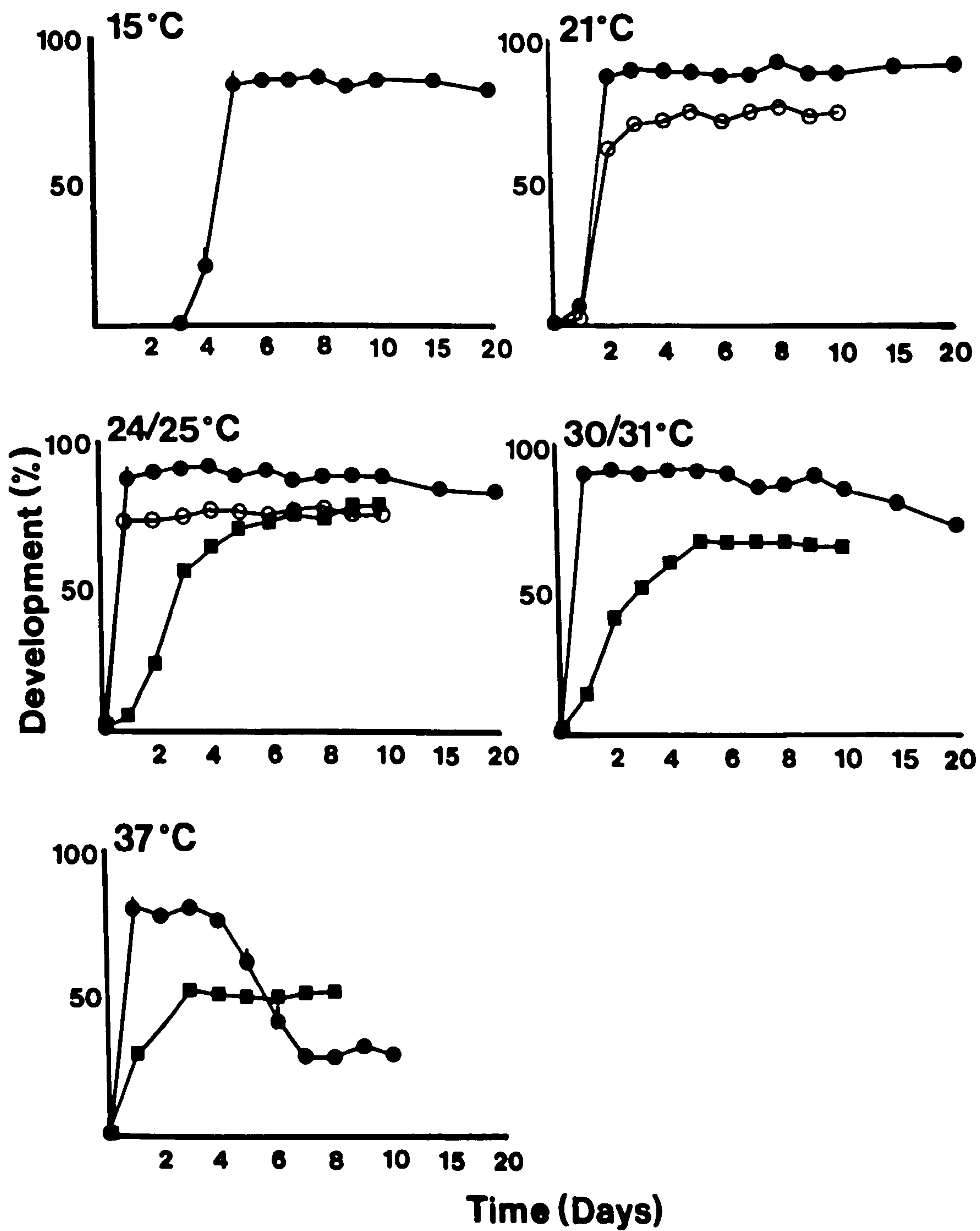
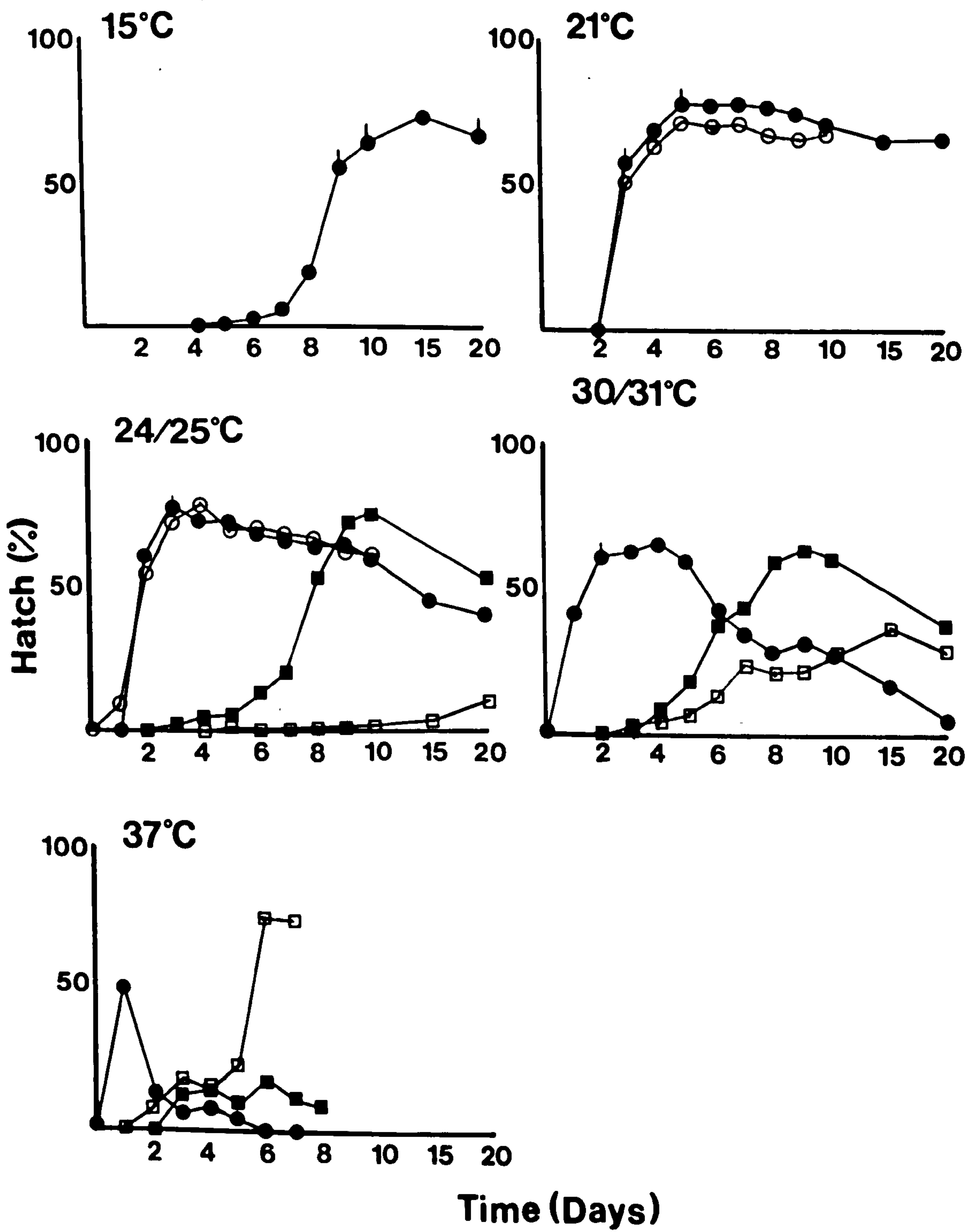


Fig.2. The effect of developmental temperature on spontaneous and stimulated hatching.

Faecal eggs were examined daily at 15°, 21°, 25°, 31° and 37°C for spontaneous hatching (□—□) and stimulated hatching (●—●). Uterine eggs were examined daily at 21° and 25°C for evidence of stimulated hatching (○—○). For each value, n=22 replicates of 30-100 eggs. Unless depicted standard error lines are covered by the symbols. The results obtained by Anya (1966a) for stimulated hatching of uterine eggs (■—■) at 24°, 30° and 37°C are also presented.



37°C plus comparable data obtained from Anya (1966a). For both spontaneous emergence and stimulated hatching the percentage hatch was calculated as described in Section 2.1.4.

a) Spontaneous emergence

Increased temperature resulted in a decrease in the incubation period required prior to the onset of spontaneous larval emergence and an increase in the number of larvae which emerged. There was no spontaneous hatch at 15°C and at 21°C and 25°C it was low, reaching 2% and 10% respectively after 20 days incubation. At 31°C spontaneous hatching commenced after 2 days and reached 40% by 9 days and at 37°C free larvae were present after 24 hours and reached 75% by 6 days. Spontaneous emergence for uterine eggs at 21°C and 25°C was equivalent to that of faecal eggs.

b) Stimulated hatching

The higher the developmental temperature the shorter the period of incubation before eggs were capable of hatching *in vitro*. At increased developmental temperature maximum levels of hatch were reached more rapidly but the percentage hatch was reduced.

Eggs incubated for 24 hours at 37°C and 31°C, and 48 hours at 25°C hatched when stimulated. The maximum levels of hatch were obtained on days 1, 4 and 3 respectively and were of the order of 40%, 65% and 80%. Hatching levels declined with increasing developmental temperatures due to larval disintegration within the eggs, 0% hatched after 6 days at 37°C, 5% after 20 days at 31°C and 40% after 20 days at 25°C.

The slower development of eggs at 21°C and 15°C resulted in an increase in the period of incubation required before hatching could be initiated *in vitro* to 3 and 5 days respectively. Maximum hatch was attained after 5 days at 21°C, it reached a level of about 80% and showed a gradual decrease to 75% by day 20. At 15°C a maximum hatch of 70% was attained after 15 days this declined slightly by the final observation on day 20. Uterine eggs required similar periods of incubation to faecal eggs at 21°C and 25°C before hatching commenced *in vitro* and they attained an equivalent percentage hatch. Data obtained by Anya (1966a) for uterine eggs developed at 24°C, 30°C and 37°C

and hatched in buffer, pH 7, at 37°C revealed considerable differences when compared to the results reported here (Fig.2). These differences are examined in the discussion.

Osmotic concentration

Fig.3. shows the effect of different osmotic concentrations on the development and subsequent hatching of *A.tetraptera* eggs *in vitro*. Development appeared normal at all concentrations and in distilled water, mean \pm SE percentage development was 95% \pm 1%. Shrinkage was visible in a proportion of the eggs incubated at the highest osmotic concentration of 1919mOsm/kg (1M NaCl), mean percentage shrinkage was 41% \pm 2.5%. Eggs incubated in osmotic concentrations of 478mOsm/kg (0.25M NaCl) or less including distilled water hatched normally, mean \pm SE percentage hatch was 91.5% \pm 1%. Fewer larvae which had developed at higher osmotic concentrations hatched *in vitro*, the mean \pm SE percentage hatch was 37.5% \pm 2% at 930mOsm/kg (0.5M NaCl) and no larvae hatched from eggs which had been incubated at 1919mOsm/kg.



From preliminary experiments on the osmotic concentration of faecal matter it was observed that faecal samples swelled and gained water in solutions of 7,500mOsm/kg or less. In a 10,000mOsm/kg solution the sample remained flaccid and weight loss occurred, which suggests that faecal osmotic concentration is between 7,500 and 10,000mOsm/kg.

Infectivity in vivo

Table 1 shows the period of incubation required at 25°C by larvae for the development of infectivity, as indicated by the presence of eggs in the host faeces following the necessary patent period. For both uterine and faecal eggs no infections developed in mice infected with 24 and 48 hour old eggs. Eggs incubated for 3 days or more produced infections in most cases; exceptions occurred in faecal eggs incubated for 3 and 7 days, in which 1 of the 2 administered doses for each day failed to produce an infection and in uterine eggs incubated for 6 days. No worms were found in the large intestine of any mice which gave negative faecal counts, including the controls:

Egg counts showed a wide range of variability between mice infected with

Fig.3.The effect of osmotic concentration on egg development and stimulated hatching.

Faecal eggs were examined after 9-10 days incubation in a range of saline concentrations for evidence of development () and subsequent hatching *in vitro* at 37°C (). For each value n=2 samples of 150-250 eggs each.

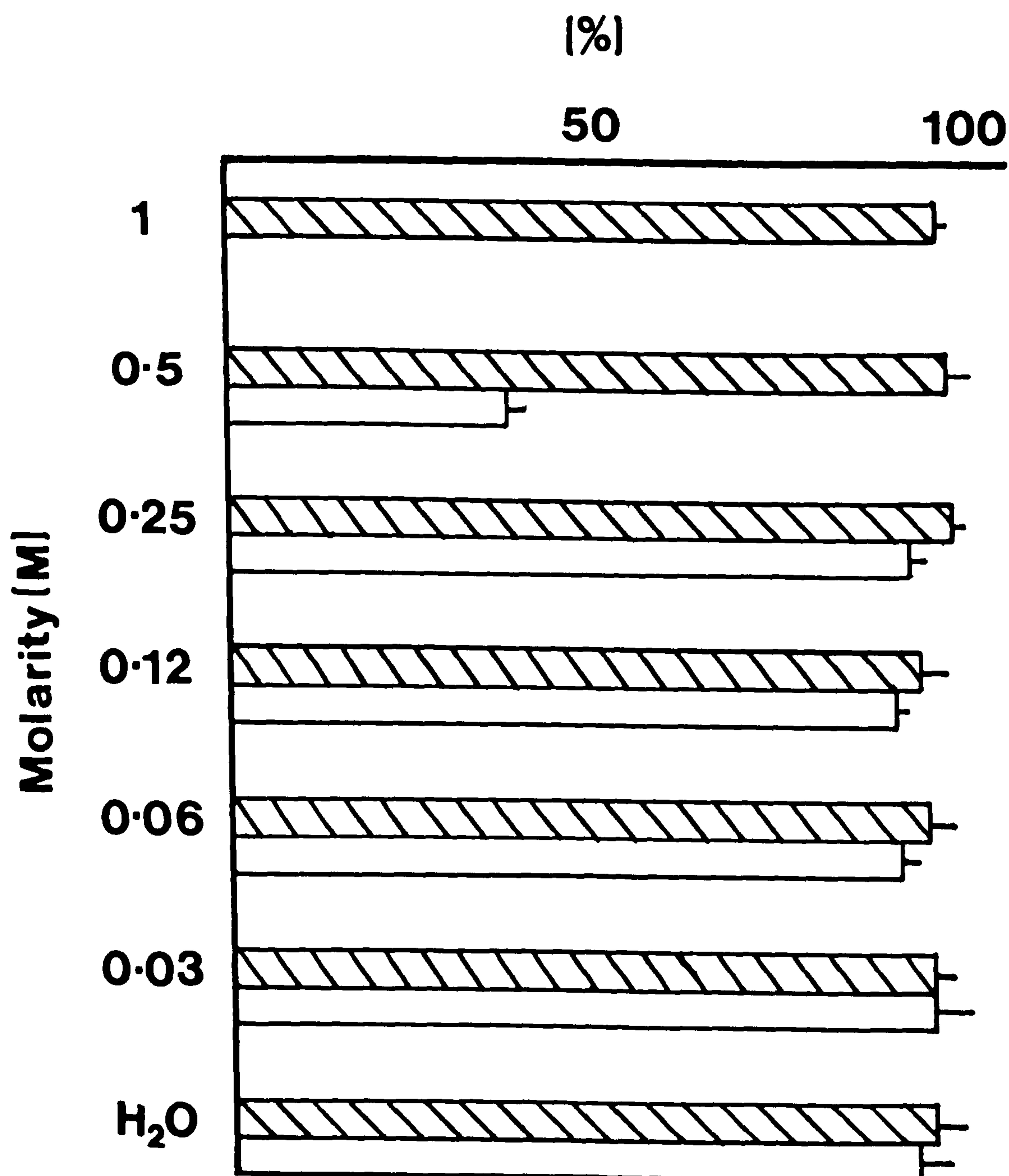


TABLE 1

The data presented below gives the mean+SE number of eggs obtained from faecal samples of mice 25-30 days post infection with uterine and faecal eggs previously incubated at 25°C for differing lengths of time. Each value was obtained from 3 egg counts.

Period of incubation for eggs prior to infection Days	Egg counts per mouse		
	Faecal eggs		Uterine eggs
1	0	0	0
2	0	0	0
3	803+23	0	634+45
4	655+57	702+20	891+35
5	473+39	912+24	589+47
6	956+12	804+45	0
7	241+86	0	497+78
8	515+63	471+67	378+44
9	909+25	600+21	758+34
10	758+98	345+57	654+27
Controls	0	0	0

eggs incubated for different periods and in repeat counts from individual mice. No correlation was observed between length of incubation of eggs and increased egg counts. The data concerning the development of infectivity disagrees with the results obtained by Anya (1966a), these differences are examined in the discussion.

3.2.4 Discussion

Temperature, humidity, oxygen and incubation media are known to be important factors in *A.tetraptera* egg development (Philpot, 1924; Deschiens, 1944; Hsu, 1951; Chan, 1953; Anya, 1965, 1966a and Wharton 1980). Anya (1966, 1966a) has presented the most detailed analysis of development and hatching of *A.tetraptera* eggs. He used eggs obtained from the uteri of adult females but his data (plotted on Figs.1 & 2) lack critical statistical analysis which makes direct comparisons difficult. The present results which are in general agreement with those previously published, differ in a number of important aspects.

Unlike most other workers eggs from both female worms and mouse faeces have been used. The differences found between the uterine egg data obtained by Anya (1966a) and faecal and uterine egg development (Fig.1) are unlikely to have been due to differences in development between the egg sources. The uterine eggs were extracted from the same source as the faecal eggs and when incubated at 21°C and 25°C showed a similar rate of development at equivalent temperatures, however the maximum development of uterine eggs was reduced to levels equivalent to those achieved by Anya (1966a). The lower maximum development levels probably reflect the fact that eggs removed from the uteri of gravid worms inevitably contained a proportion of unfertilised and immature eggs that were incapable of embryonation.

The effect of temperature on both faecal and uterine eggs was to increase the rate of larval development with increased temperature (Fig.1). However the number of larvae which disintegrated also increased at 31°C and 37°C. Larval degeneration and loss of larval motility have been reported in eggs incubated at 37°C (Anya, 1966a and Hsu, 1951). This may indicate the upper temperature range for the distribution of the parasite, however no literature is available on

the geographical range of *A.tetraptera*

The sampling frequency, with examination of the eggs every 24 hours was insensitive and at temperatures over 21°C the rate of development was such that peak development had been reached prior to the first sample being inspected.

In most previous studies (Philpot, 1924; Deschiens, 1944; Hsu, 1951, Chan, 1953; Anya, 1965 and Wharton, 1980), larvae were found to take 24-65 hours to develop in the egg at temperatures of 20-30°C, this supports the present results but also conflicts with Anya (1966a). Although the rates of development obtained by Anya (1966a) for uterine eggs incubated at 24°C, 30°C and 37°C increased with increasing temperature the rate of development was substantially reduced compared with either faecal or uterine eggs in the present work. The incubation times taken to reach maximum development were 8-9 days at 24°C, 4-5 days at 30°C and 2 days at 37°C (Anya, 1966a).

Spontaneous hatching was negligible from 15-25°C however spontaneous emergence of larvae increased at higher temperatures (31°C and 37°C) and accounted for a considerable proportion of the larvae which finally emerged (Fig.2). From five days onwards spontaneous hatching was greater than stimulated hatching at 37°C, suggesting that temperature is a major factor in activating hatching.

Anya (1966a) does not report spontaneous emergence at any of the temperatures he investigated. However he maintained his cultures according to the method described in Anya (1966a) where allowances were made for background hatching whilst estimating percentage hatch in eggs. Anya (1966a) only stimulated hatching in eggs which had previously shown no evidence of hatching when centrifuged in the hatching media. He found controls which had been incubated for 9-10 days at 24°C showed background hatching of between 2% and 5% which corresponds to levels found at 25°C in the present data (Fig.2). Hsu (1951) also found larvae began emerging from eggs after 1 month's incubation in water at 28°C and, after 2 month's incubation most of the larvae had hatched. It is likely that this temperature was sufficient to slowly stimulate hatching. Background hatching is investigated in detail in Section 4.3.

The higher the developmental temperatures, the shorter the period of

incubation required by eggs before they were capable of hatching when stimulated *in vitro* and before maximum levels of hatching were attained (Fig.2). At 31°C and 37°C levels of hatching began to decline after 5 and 2 days respectively due to larval degeneration within the eggs. Anya (1966a) who incubated uterine eggs at 24°C, 30°C and 37°C also observed that eggs required a shorter period of incubation at higher developmental temperatures before *in vitro* hatching occurred when stimulated. However he found a period of incubation during which the larvae though developed were incapable of hatching (Figs.1 & 2), the time lag between maximum development and maximum hatching was 3 days at 24°C, and 4 days at 30°C. These compared to 2 days at 25°C and 1 day at 31°C in the present work (Figs.1 & 2). Equivalent levels of hatching were obtained for both sets of data at 24–25°C and 30–31°C, at 37°C the maximum level of hatching was reduced by 30–35% in Anya's data (Anya 1966a).

It is difficult to provide a satisfactory explanation for the fact that Anya's results differ both from the present data and those of other authors. The method of data collection and presentation of his results precludes statistical comparisons. In the present study a large number of samples were analysed for each point in an attempt to increase the reliability of the data. The small variances obtained allows them to be regarded with some confidence. The temperatures used were not exactly the same as Anya but differed by only about 1°C which is unlikely to cause the wide differences found. A further possibility is that eggs from different *A.tetraptera* strains, selected by different regimes in individual animal houses in the isolation of stable host colonies, differ markedly in their developmental and hatching characteristics. There is, however, no literature on strain differences in *A.tetraptera* and preliminary observations on eggs from a number of independent sources have revealed no differences.

Hsu (1951) reported that larval development was apparently normal in osmotic concentrations of 65–275mOsm/kg (0.25–0.85% NaCl solutions). However he made no observations on their ability to hatch. This deficiency has been rectified and Fig.3 indicates that while embryonation occurs at the osmotic concentrations investigated (1919mOsm/Kg or less), the ability to hatch when stimulated *in vitro* decreases at osmotic pressures above about 500mOsm/kg. These results and the preliminary observation that mouse

faeces have an osmotic concentration of between 7,500 and 10,000mOsm/kg, suggest that the optimum development of infectivity found by Anya (1966a) of 9-10 days at 25°C would expose the eggs to considerable osmotic stress. However, the degree of osmotic stress experienced by the eggs in the faeces is likely to be dependent on the faecal moisture content. Thus eggs developing in relatively dry faecal pellets may be exposed to less osmotic stress than indicated in the preliminary experiment. The findings of the present data, which indicate that eggs can sustain infections after only 3 days incubation at 25°C would allow reduced exposure of the eggs to osmotic stress while infectivity is obtained.

An examination of the period of incubation required by larvae for the development of infectivity to mice, as indicated by the presence of eggs in faeces after the necessary patent period (Table 1), suggests that both faecal and uterine eggs are capable of producing infections after 3 days incubation at 25°C. These results differ from the findings of Anya (1966a) who examined the number of larvae recovered from mice infected with eggs cultured at different temperatures for varying lengths of time. He found that at a comparable temperature (24°C), a 5 day incubation period was necessary before infection could occur. He also observed that heavier infections resulted as the period of incubation of eggs prior to administration increased. This trend was not apparent in the egg counts made from mice infected with increasingly older eggs. Thus the suggestions made by Anya (1966a) following his deductions from the studies of Philpot(1924), Hsu(1951) and Chan(1955) that a period of incubation of at least 5-6 days at 25°C was necessary for infection to result is not verified.

Under the conditions present in the animal house, *A.tetraptera* eggs experience a developmental temperature of 22°C. At this temperature eggs need to develop infectivity within 7 days (the cage cleaning cycle) in order to maintain natural infections. The findings of the experiments described in this chapter show that at 21-25°C, maximum development is reached in 1-2 days, maximum hatching occurs in 3-5 days and eggs are capable of sustaining infections after 3 days incubation at 25°C. Anya (1966a), on the other hand found maximum infectivity occurred after 9-10 day incubation period at 24°C. He found only about 25% of the larvae present were capable of hatching *in vitro* and initiating infection *in vivo* after a 7 day incubation period.

Unfortunately Anya (1966a) does not mention the natural environmental conditions of the egg populations he examined, but a weekly cage cleaning cycle would not allow optimum infectivity in the majority of eggs and it seems unlikely that a stable parasite population could be naturally maintained.

3.3 Individual egg development

3.3.1 Introduction

Questions which have arisen from the study on population egg development (Section 3.2) are investigated in detail in this chapter at the individual egg level. Time lapse video micro recordings have been used for much of the study. Investigations undertaken include examining i) the pattern of larval embryonation, ii) larval development and eggshell structure for differences in faecal and uterine eggs under various conditions, and iii) the effect of increased temperature on rates of development. The reasons prompting this study are given below.

Firstly, although aspects of the life cycle of *A.tetraptera* have been studied previously (Section 3.1), little mention has been made of the pattern of embryonation of the developing larva within the egg. Philpot (1924) published three illustrations of stages of egg development from eggs incubated in water at 25°C for 24, 45 and 68 hours. Anya (1965) mentions that embryonation had reached the 65 cell stage when eggs were recovered from host faeces. Therefore the pattern of larval embryonation in the egg was examined in order to determine whether it followed the general pattern of nematode development, as reviewed by Nigon (1965), Hope (1974) and Ehrenstein and Schierenberg (1980).

Secondly, it was deduced from the controlled experiments on egg population development that the contradictions which arose between Anya's results (Anya 1966a) and the collected data were not due to any apparent differences between uterine and faecal eggs. However the parameter used for assessing development, that is the presence of elongated larvae, was too insensitive to detect any subtle variations in development which might be present between the uterine and faecal eggs.

Thirdly, the frequency of data observations made in Section 3.2.2. was not accurate enough to determine any differences which might exist in rates of development of eggs incubated at increasing temperatures over 25°C and fourthly, the observation in Section 3.2.3. that an osmotic concentration of

1119 mOsm/Kg caused shrinkage of developed larvae in uterine and faecal eggs did not show whether the larvae experienced the same rate of shrinking and thus the same permeability change in the two types of eggs. Therefore the effect of the above mentioned conditions on faecal and uterine eggs were examined in detail using video analysis.

Differences have been observed in faecal and uterine eggs by previous authors (Monne and Honig, 1954b; Wharton, 1979b). Monne and Honig reported that faecal eggshells were brown and insoluble in all reagents except sodium hypochlorite. Wharton (1979b) observed that uterine eggs were colourless when dry and soluble in acids, alkalis and various enzymes. Both Anya (1964b) and Wharton (unpublished observations, cited Wharton, 1980) attempted to demonstrate that the brown colouration of the faecal egg was the result of quinone tanning of the eggshell on passage through the host gut. However no evidence was found of the presence of phenols and/or phenolases.

Differences in the faecal and uterine eggs of several nematodes have been reviewed by Fairbairn (1957) and are described in Section 3.1, where it has been suggested that the brown appearance of the eggshell is a result of bile staining and may afford protection against ultraviolet light. To examine whether this is the case in *A.tetraptera* eggs, uterine and faecal eggs were exposed to ultraviolet light under controlled conditions.

It was hoped that a comparison of uterine and faecal eggs at the ultrastructural level would reveal any differences in eggshell structure between the two eggs. Unfortunately no satisfactory transverse sections were obtained of uterine eggs for examination by the transmission electron microscope. However electron micrographs obtained from faecal egg sections were compared with plates of transverse sections of uterine eggs from an ultrastructural study by Wharton (1979b). The structure, chemistry and formation of the eggshell of *A.tetraptera* have been examined in detail by Anya (1964a & b) and Wharton (1979a & b). These are discussed in Section 3.1. with reference to the oxyurid and nematode eggshell in general, as reviewed by Wharton (1980a & b), Anya (1976), Bird (1971), Rogers (1962), Fairbairn (1957) and Christenson (1950).

Qualitative observations on the egg size of uterine and faecal eggs in Section 3.2. suggested that uterine eggs were smaller than faecal eggs.

Wharton (1979b) found the average dimension of uterine eggs to be 45 x 90 microns. A comparison of the measurements of both types of eggs was undertaken to assess whether any difference in egg size was present.

3.3.2 Materials and methods

Egg size

Uterine and faecal eggs were extracted as described in Section 2.1.2. For each type of egg a minimum of 50 eggs in 0.3 ml. distilled water were suspended in 1.5 mls. saturated sodium chloride in a counting chamber. The chamber was then placed on the microscope stage and left for one minute to allow the eggs to float to the surface. The measurements for each batch of eggs were completed within as short as period as possible (less than 5 minutes) to minimise any effect the saturated sodium chloride may have on the egg volume. Using a Vickers image shearing module and "Shear" computer programme (E.R.C.C; Edinburgh University) loaded into a BBC microcomputer, the eggs were measured by shearing along the length of the egg, recording it, rotating the egg through 90 degrees, shearing across it's breadth and recording this dimension. The data obtained was filed on computer and analysed using further software, in this case the mean length and breadth data were obtained fom the programme "EGGRAPH" (David Smith, Moredun Institute). The results are presented in Fig.4. and Table 2.

Individual egg development

Uterine and faecal eggs were collected as described in Section 2.1.2, except the faecal eggs were obtained from two hour old samples. The time-lapse video recording techniques used to measure egg development were devised by Matthews (1985) and are described in Section 2.1.6. The eggs analysed were incubated in distilled water and egg development was recorded in 80 hour mode. Development was observed at 25°C for both uterine and faecal eggs and at 37°C for faecal eggs only. The results are presented in Table 3. Light micrographs were taken of faecal eggs at various stages of development at 25°C (Plates 1 & 2).

Larval shrinking

Uterine and faecal eggs were obtained as described in Section 2.1.2. Larval shrinking was recorded using time lapse video as described in Section 2.1.6. For each type of egg a sample of 15-20 eggs was incubated in distilled water at 25°C on the warm stage until larval activity had started. The water was then

replaced with 1M NaCl solution and the eggs recorded in 80 hour mode with 3 minutes recording at "normal speed" using the automatic alarm facility.

Ultra-violet light

Faecal and uterine eggs were collected as described in Section 2.1.2. From each batch of eggs, six samples with a sample size of 200–300 eggs per faecal eggs and 50–150 eggs per uterine eggs were prepared for incubation as described in Section 2.1.3. but incubated at 25°C, the constant temperature of the rooms where the treatments were conducted. Treatments included ultraviolet light, artificial light and no light. For each treatment a daily routine of 12 hours light/12 hours dark was imposed. Ultraviolet light, 360–370nm was provided by a high pressure U-shaped quartz mercury arc tube in an ultraviolet lamp (Hanovia Technical Lamp, Engelhard Ltd.), artificial light, 575–585nm from the overhead strip light (Streamlite popular, white 35 lamps, Philips Ltd.) present and a control of constant darkness as occurs during normal incubation in the incubator. Two samples each of faecal and uterine eggs were incubated per treatment and for the control. Eggs were observed daily for evidence of development and subsequently examined for hatching at 37°C if larval development occurred (Section 3.2.).

Ultra-structural studies

Unembryonated faecal and uterine eggs were collected and prepared for observation by transmission and scanning electron microscopes as described in Section 2.1.2. and 2.1.5. respectively, the eggs were squashed to allow full penetration of the fixative. Problems were encountered in obtaining satisfactory transverse sections of uterine eggs because a) the "sticky" outer coat caused the eggs to adhere to the handling equipment at various stages of the preparation procedure and b) the squashing of the eggs to allow fixative penetration so disrupted the eggshell wall in uterine eggs that sections obtained were not comparable with those of faecal eggs. Electron micrographs of the ultrastructure of the faecal egg and the surface structure of both types of egg are shown in Plates 3 and 4, respectively.

3.3.3 Results

Egg size

The average dimensions of the faecal and uterine egg data, that is their mean length and breadth are given in Table 2. A scatter diagram plotting the range of variation of the egg data collected is displayed in Fig.4. Both the scatter diagram and the mean dimensions suggest that the faecal eggs are slightly larger than the uterine eggs. Unfortunately, the computer program failed to relay the individual egg measurements, preventing further comparison of the data statistically.

Individual egg development

The recorded tapes were analysed at accelerated playback speed, which allowed larval development within the eggs to be viewed continuously.

The faecal and uterine eggs were at various stages of blastula development or early gastrulation (Plate 1a) when incubated at 25°C. By the process of gastrulation an elongated cylindrical larva formed. Movement began with sudden twitching of the larvae which increased in frequency until the whole larva was capable of rotating on its own axis. No further elongations of the larva occurred after this point, the larva appeared to be developed and to have undergone a moult as suggested by the presence of a sheath (Plate 1b & 2).

The heterogeneity^e of the samples available made it essential to establish identifiable points of development for comparative purposes between the faecal and uterine eggs. Three parameters were easily distinguishable, i) start of elongation to form cylindrical larvae, ii) end of extension of cylindrical larvae and iii) first larval activity. These parameters were measured for each group of eggs analysed and all three were found to be suitable for comparing larval development. In this experiment the occurrence of the first larval movement was the parameter used for comparing the development of faecal eggs at 25°C and 37°C and uterine and faecal eggs at 25°C (Table 3). It was observed that the development time of the faecal eggs decreased with increasing temperature. A comparison of the mean \pm SE development of 20 eggs at 25°C and 15 eggs at 37°C revealed no difference between the two groups

Fig.4.Scatterdiagram of faecal and uterine egg measurements.

The width and length of faecal (O—O) and uterine (x—x) eggs are plotted.
For each type of egg,n=50.

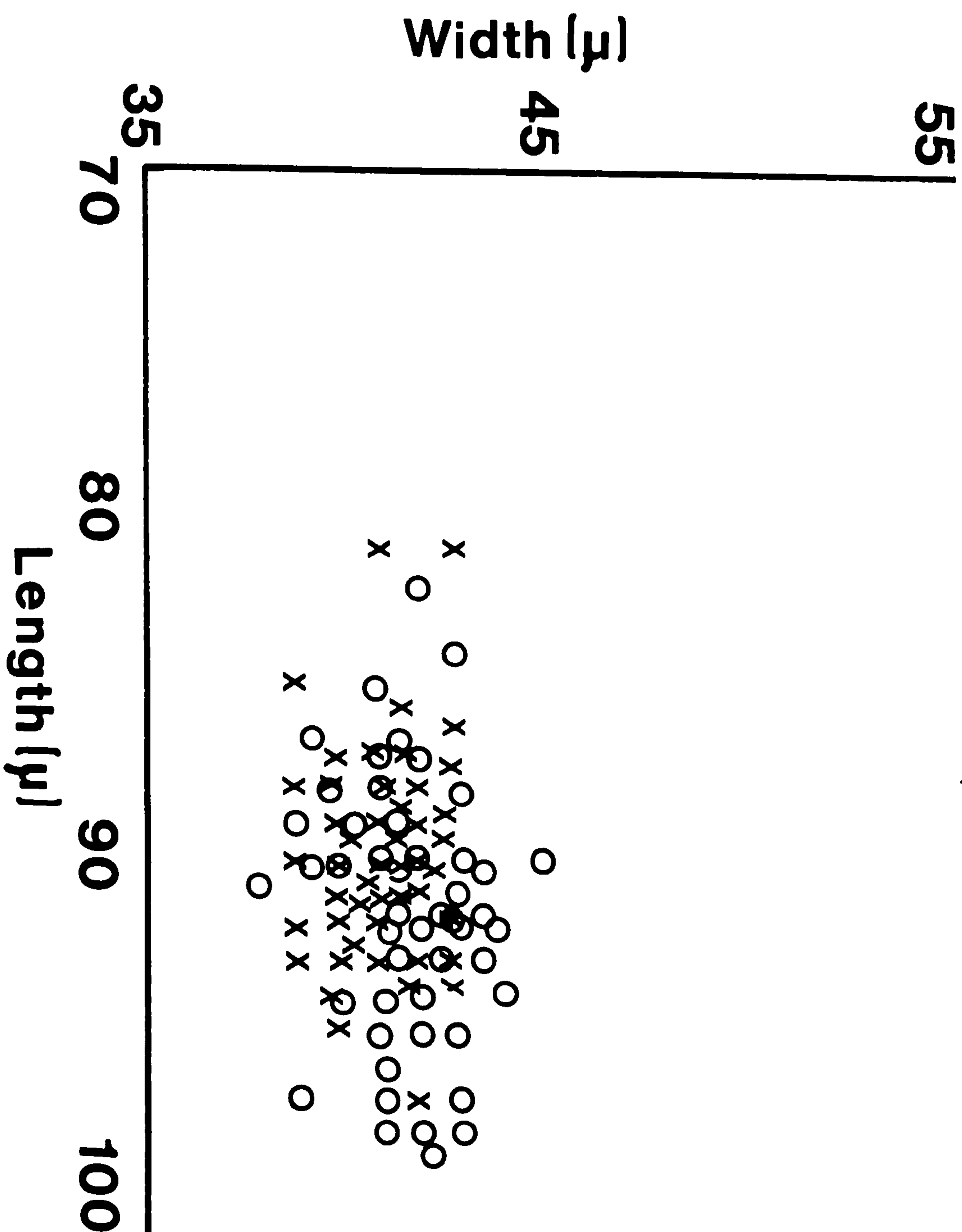


TABLE 2

The data presented below gives the mean length and width of uterine and faecal eggs. Each value was obtained from 50 eggs.

Egg Type	Length (microns) X	Width (microns) X
Faecal	92.22	41.5
Uterine	90.24	40.72

TABLE 3

The data presented below gives:- i) the mean+SE time (Hr.Min.) to first larval activity at 25°C and ii) the sample size (n).

Egg Type	Temperature	
	25°C Time	37°C Time
Faecal	24Hr.59Min.+55Min. n=20	11Hr.48Min.+36Min. n=15
Uterine	30Hr.41Min.+1Hr.9Min. n=20	

of faecal eggs. On comparing the mean \pm SE development times of uterine and faecal eggs, 20 eggs each, at 25°C, no difference was found between the two types of eggs.

For both uterine and faecal eggs, the occurrence of the three parameters relative to one another within each egg were similar, independent of the stage of embryonic development at initial incubation. A comparison of the mean \pm SE formation time of cylindrical larvae and the mean \pm SE time between larval formation and larval activity between both types of egg, revealed no difference. However a comparison of the mean \pm SE time required until the commencement of larval elongation in both faecal and uterine egg revealed that uterine eggs approx. 6 hours longer to develop than faecal eggs at 25°C.

A scattergram (Fig.5) plotting larval activity and initiation of elongation of larvae, gives a visual interpretation of the distributional spread of the data collected. Three centres of distribution are apparent, i) faecal eggs at 37°C, ii) faecal eggs at 25°C and iii) uterine eggs at 25°C with each showing a greater spread in distribution respectively. Some overlap occurred between faecal and uterine egg development times at 25°C, 30% and 35% respectively, however no overlap occurred between the development time of faecal eggs at 37°C and faecal and uterine eggs at 25°C.

At 25°C, 9% of faecal eggs and 16.6% of uterine eggs remained undeveloped and at 37°C, 22% of faecal eggs were unembryonated after incubation.

Larval shrinkage

The mean \pm SE rate of larval shrinkage in uterine and faecal eggs, incubated in 1M NaCl at 25°C is compared in Table 4 and the onset of shrinking in Table 5. No difference was found between the onset of larval shrinking, the reduction in larval length and the rate of shrinkage, which occurred between the two batches of eggs.

Ultraviolet light

The results of the effect of various light regimes on the development of uterine and faecal eggs as shown by the presence of larvae and their subsequent hatching *in vitro* when stimulated (Section 2.1.2) are displayed in

Fig.5.Scatterdiagram of uterine and faecal egg development, using behavioural parameters.

For each type of egg, 2 behavioural parameters were recorded:- i) time to first larval activity and ii) time to formation of cylindrical larvae. Faecal egg development was measured at 25°C (⊕—○) and 37°C (▲—▲). Uterine egg development was measured at 25°C (●—●). For each type of egg, n=15-20.

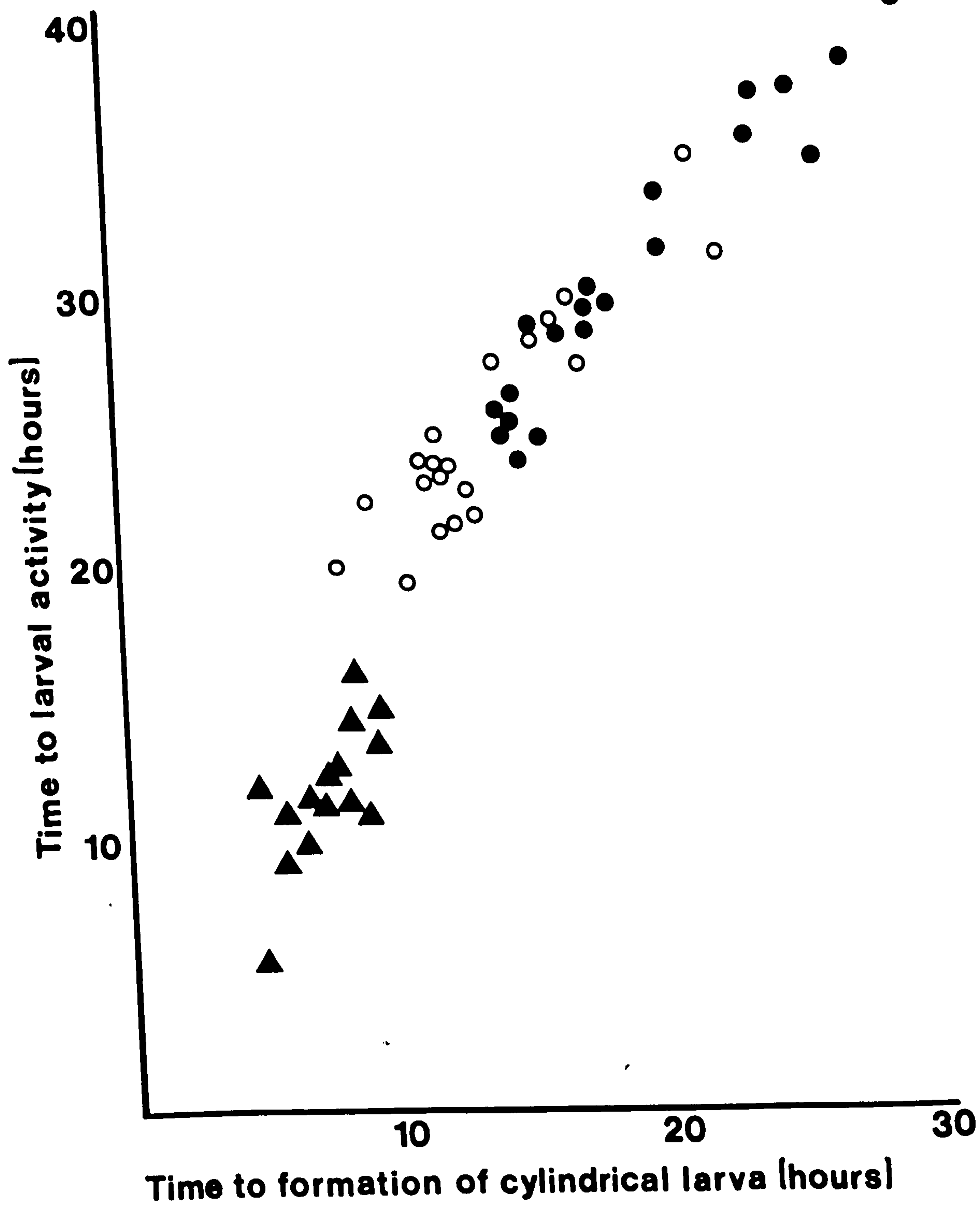


TABLE 4

The data presented below gives the mean+SE decrease in length (μ) per minute of larvae incubated in 1MNaCl (1919mOsm/Kg) at 25°C. Each value was obtained from 8 eggs.

Egg Type	Rate of shrinking of larval length per minute
Faecal	6.11 \pm 0.43
Uterine	5.66 \pm 0.54

TABLE 5
 The data presented below gives the mean+SE incubation time (Mins.) before larval shrinking commenced (Hr.Min.) in faecal and uterine eggs incubated in 1MNaCl (1919mOsm/Kg) at 25°C. Each value was obtained from 8 eggs.

Egg Type	Time to onset of larval shrinking
Faecal	132 ₂₅
Uterine	162 ₂₉

TABLE 6

The data presented below gives:- i) the mean+SE number of eggs which developed after exposure to different light regimes for 1 week at 25°C and ii) the mean+SE number which subsequently hatched when incubated in 0.05M Bicine buffer (pH 8.5) at 37°C overnight. Data was obtained from 6 replicates of 200-300 faecal eggs and 50-150 uterine eggs.

Treatment	Development (%)		Hatching (%)	
	Faecal Eggs	Uterine Eggs	Faecal Eggs	Uterine Eggs
Dark	97+1	61+10	92.5+2	85+2
Artificial light	91.5+2	70+7	82.5+1	80.5+2.5
Ultraviolet light	11+2.5	0	10+2	0

Table 6. Artificial light had no effect on the development and subsequent hatching at 37°C of uterine and faecal eggs when compared to the controls (darkness). However ultraviolet light had a marked effect, causing much reduced development and hatching in faecal eggs (about 80%) and no development and thus no hatching in uterine eggs.

Ultrastructural studies

The transverse section of the faecal eggshell (Plate 3a & b) shows there is no difference in the number and structure of layers present in the eggwall when compared to the uterine eggshell section displayed in Plate 1b, Wharton (1979). The scanning electron micrograph of the uterine egg (Plate 4b) shows particulate material adhering to its outer surface whereas the faecal egg surface (Plate 4a) is comparatively clean. The external uterine layer of the uterine egg appears to be less developed than the faecal egg as the definition of this layer is reduced at the uppermost end of the egg as indicated by the arrow.

3.3.4 Discussion

The data collected from the study of the effect of temperature on population egg development and larval viability (Section 3.2.), provided observations on general trends present and allowed adequate comparisons to be made with Anya's data (Anya 1966a). It confirmed the results of the preliminary experiments which suggested that the development of infectivity was significantly faster than Anya had previously suggested. However the method and frequency of the data collection was limiting, preventing a detailed study of the pattern of larval embryonation in uterine and faecal eggs and the rate of development of eggs at temperatures over 25°C. The observations on egg population development inevitably involved heterogeneous samples due to the differential maturity within the batches of uterine and faecal eggs examined. Therefore to standardize results large numbers of replicates were necessary to reduce the variation present.

The use of time lapse video micro recording in the present study made it possible for gradually changing processes in the egg to be viewed continuously, enabling detailed observations to be made on larval development and behaviour at the individual egg level. The adaption of behavioural parameters allowed direct comparisons to be made between individual eggs under controlled conditions and accurately measured the heterogeneity of the sample analysed.

Video recordings of rates of development of faecal eggs at 25°C and 37°C revealed a shortened development time at the higher temperature (Table 3, Fig.5), as expected but not manifested in the egg population study (Section 3.2.). Although the development time was halved at 37°C when compared to 25°C, the percentage of eggs which failed to develop doubled, supporting the suggestion made in the egg population study that *A.tetraptera* may be limited in its distribution to temperate regions. From observations on uterine egg development at 25°C, uterine eggs were generally at an earlier stage of development than faecal eggs, taking a mean time of 5 hours and 40 minutes longer to obtain larval activity. This difference in development time of faecal and uterine eggs is not sufficient to account for the delay in development of 7-8 days which occurred between Anya's uterine egg data (Anya 1966a) and the uterine and faecal egg data presented in the population study at 25°C

(Section 3.2.). Thus the other possibilities expressed in detail in Section 3.2. which may account for differences in the egg data must be considered. They include i) inaccurate temperature measurements, ii) differential assessment of development due to the qualitative parameters used and iii) the use of eggs from different worm strains.

The pattern of larval development in the eggs was consistent with that found for nematodes in general, excepting that development ceased at a stage equivalent to the tadpole stage of many nematodes, continuing once infection resulted. Nematode development has been reviewed by Nigon (1965), Hope (1974) and Ehrenstein and Schierenberg (1980). The emerging larva is enclosed by a membrane which may either represent a moulted cuticle or the inner membrane of the eggshell. The latter suggestion seems more likely.

Although the pattern of embryonation is similar in uterine and faecal eggs, it has been observed that faecal eggshells are brown and more resistant to acids, alkalis and various enzymes than the transparent uterine eggshells (Monne and Honig, 1954b; Wharton, 1979), which may indicate structural differences. One explanation for the development of the resistant brown shell is quinone tanning, however neither Anya (1964b) nor Wharton (unpublished observation, cited Wharton 1980) have found any evidence of this process occurring.

An attempt was made to examine the structure of both unembryonated uterine and faecal eggs, unfortunately due to difficulties encountered in sectioning individual uterine eggs only faecal sections were obtained. However Wharton (1979b) had previously examined unembryonated uterine eggs *in situ* successfully, enabling a comparison to be made between both types of eggs. An examination of the electronmicrographs in Plate 3 of the present study and Plate 1b-d (Wharton, 1979b) revealed no apparent differences in the number and structure of the various layers of either egg.

As no structural differences were observed between the two types of eggs at the level of magnification examined, larval shrinking was measured in the eggs using video analysis (Table 4). At an osmotic concentration of 1919mOsm/kg the rate of larval shrinking was equivalent suggesting the permeability of the eggs to water was equal. Therefore the differing abilities of the eggs to resist acids, alkalis and various enzymes appears not to be due to

any difference in the permeability of the eggs at the structural level examined, or at the surface level described below.

A comparison of the exteriors of uterine and faecal eggs (Plate 4a & b), revealed that the surface of this particular uterine egg was less sculptured at one end. This may indicate incomplete shell formation due to its position *in utero* when collected. The uterine egg (Plate 2b) examined by Wharton (1979b) probably represents a more mature egg as the eggshell appears equally sculptured to that of the faecal egg (Plate 4b). Particulate material adheres more readily to uterine eggs than faecal eggs as revealed in Plate 4a, and Plate 2e of Wharton's study. The disappearance of this adhering property of the outer coat of the uterine egg on passage through the mouse large intestine suggests some change has occurred in the composition of the faecal eggshell.

In a review by Fairbairn (1957), it has been suggested that the brown appearance of faecal nematode eggs is a staining effect of bile on the eggshell (Section 3.1.). Whether bile would still be sufficiently concentrated after reaching the mouse large intestine to stain uterine eggs released by the gravid worm is unknown. However some form of staining must occur in the mouse intestine, as indicated by the brown colouration of the host faeces. From personal observations on eggshell staining, it has been noticed that the uterine coat of the immersed egg rapidly incorporates the colour of the solution. Thus the change in colour of the eggshell on passage through the host may be a staining effect of the bowel contents as opposed to a chemical change in the shell.

It has been postulated in Fairbairn (1957) that the brown colouration observed in various nematode eggs may protect larvae against ultraviolet light. The data presented in Table 4 suggests that the faecal eggshell may offer some protection against ultra-violet light as 20% of the eggs developed compared to 0% development in the uterine eggs. Under normal development such prolonged exposure to ultraviolet rays is unlikely and the faecal egg may adequately protect the egg for shortened periods. Exposure to sunlight in eggs released under natural conditions would depend on the prevailing climate and location of faecal deposits. Under laboratory conditions, eggs are exposed to an artificial light regime in the animal house which was shown to have no

effect on larval viability. The adaption of the worm to animal house conditions allows it to avoid the rigours of the natural environment.

CHAPTER 4 HATCHING

4.1 Literature Review

This review examines the literature concerning nematode hatching, including nonspecific and specific stimuli, stages prior to and during the hatching process, including the possible existence of a diapausal stage, and proposed mechanisms. Various aspects of hatching have been reviewed extensively and are mentioned where appropriate. This review will therefore concentrate on features considered to be particularly relevant to this thesis.

Spontaneous hatching is found in many free-living nematodes and plant and animal parasitic species with a free living phase in their life cycle. Emergence is not completely independent of the surrounding environment, as temperature, moisture and aeration all affect hatching. The influence of these factors on the development and emergence of "spontaneously" emerging nematodes are reviewed by Evans (1974) and Jones (1975a) among others. These juveniles can often emerge over a wide range of temperatures, although the time required for development prior to emergence decreases with increasing temperature. For example hatching occurred between 10°C and 35°C for free living species of the Diplogasterinae (Pillai and Taylor, 1968), between 17°C and 38°C for *Ancylostoma* species of hookworm (Matthews, 1985), between 4°C and 38°C for animal parasitic species of sheep (Crofton, 1965) and between 15°C and 35°C for plant parasitic *Meloidogyne* species (Bird & Wallace, 1965). Hatching of the plant parasitic nematodes, *M.naasi* and *H.avenae* and the animal parasitic nematode, *N.battus* (Evans, 1974) require a period of chilling to stimulate hatching. Many plant parasitic eggs including *Globodera* species and *Heterodera* species, (Clarke & Perry, 1977) and animal parasitic eggs including *Ancylostoma* species (Smyth, 1976) and parasitic nematodes of sheep (Crofton, 1961), are adapted to hatching at local climatic temperatures. Seasonal effects, in terms of diapause are discussed below.

The effects of osmotic stress on the emergence of many nematode species has been examined. The effect has been shown to be reversible in the plant parasitic species *H.scachtii* (Wallace, 1955), *M.javanica* (Wallace, 1966),

H.oryzae (Reversat, 1975) and *G.rostochiensis* (Clarke *et al*, 1978) and in the animal parasitic species *A.suum* (Clarke & Perry, 1980). Dropkin *et al* (1958) demonstrated reversible inhibition of hatch by osmotic concentration in *G.rostochiensis*, *M.javanica*, *M.arenaria* and *D.dipsaci* and found increasing concentration resulted in increased time of recovery. Larval movement was observed to cease within the egg while inhibition was in effect. Wilson (1958) found a reduction of hatching occurred in *T.retortaeformis* with increasing solute concentration. He also observed that weak electrolyte solutions depressed the hatching rate further, the rate being dependent on the motility of the slowest ion. Croll (1974) observed that development of motile larvae of *N.americanus* was unaffected by the osmotic pressure of the environment but emergence was inhibited by osmotic stress and therefore dependent on the external environment. Matthews (1985) however found increasingly hypertonic solutions directly affected the behaviour and development of 2 species of *Ancylostoma* after the active stage had been obtained within the egg. He found no correlation between reduction in hatch and ionic mobility as observed by Wilson (1958). The effect of osmotic stress on the emergence of these nematodes indicates increased permeability of the eggshell prior to emergence.

Egg development, previously³ reviewed in Section 3.1., is dependent on an aerobic environment. Oxygen has been shown to be essential for several "spontaneously" hatching nematodes including freeliving species (Nickandrow & Blake, 1972), plant parasitic species (Wallace, 1973) and animal parasitic species (Matthews, 1986). The "infective" species, *G.rostochiensis* (Atkinson and Ballantyne, 1977a) and *A.lumbricoides* (Passey and Fairbairn, 1955) showed an uptake of oxygen during the hatching process.

There is strong circumstantial evidence for the existence of a diapause stage in plant and animal parasitic larvae in eggs or free on soil and pasture. Direct evidence has been established in only a few nematodes, *G.rostochiensis* (Evans, 1982, Hominick *et al*, 1986a & b), *H.avenae* (Banyer & Fisher, 1971a & b; Fisher, 1981 and Rivoal, 1978, 1979 & 1983), *M.naasi* (Franklin, 1971; Ogunfowora & Evans, 1971 and Gioris, 1977). Less defined evidence comes from other heteroderiid species (Perry, 1987) and the animal parasitic species *N.battus* (Parkins, 1972, cited Evans, 1974). A common feature of the diapause phenomenon is the requirement of a period of chilling after embryonic and



larval development within the egg to break the diapause and enable hatching to proceed. A temperature in the region of 10°C for about 8 weeks allowed most eggs to hatch when placed at 20°C. Evidence for the existence of a diapause in nematodes has been reviewed by Evans & Perry (1976). They regarded diapause as a type of dormancy:

...."the condition in which development has been arrested and cannot be resumed until specific requirements have been satisfied, even if favourable conditions return."

Evidence obtained from studies on *H.avenae* (Banyer & Fisher, 1971a & b) indicate that diapause is obligatory in juveniles of this species. This has not been established in other diapausing species, in which case environmental factors may be the cause. Environmental factors have been observed to act on the adult nematode producing the egg. Studies on diapause in *G.rostochiensis* (Evans, 1982 and Hominick *et al* , 1986a & b) suggest that signals passed from the plant to the nematode during the growing season influence diapause during cyst production. It has been demonstrated that daylight acting on the host plant influences the hatching of juveniles through its effect on the developing females. The influence of photoperiod on the hatching of juveniles via the host plant and female nematode has also been demonstrated in *M.javanica* (Bird *et al* (1980). Environmental factors may also act on the juvenile within the egg. Parkin (1972) reported that, unembryonated *N.battus* eggs removed from faeces, developed and subsequently hatched in water without chilling, whereas embryonated eggs recovered from faeces required a period of chilling prior to emergence.

Studies on diapause in populations of *H.avenae* (Banyer & Fisher, 1971a & b and Rivoal, 1978, 1979 & 1983) found in different climatic regimes of the southern and northern hemisphere have shown it to be synchronized with the host life cycle and prevailing climatic regime such that emergence occurs when conditions are favourable. If conditions become unfavourable such as the occurrence of high temperatures following a period of chilling, a new diapause can be induced.

Perry (1987) has envisaged that the cessation of development following the induction of diapause in plant nematodes is stopped endogenously or by the action of environmental token stimuli and resumed after a time interval has

allowed the breakdown of inhibitors or the synthesis of hormones. The control of such a mechanism may be similar to that hypothesised by Rogers & Petronijevic (1982) and Petronijevic *et al* (1986) in moulting nematodes.

Gooris and D'Herde (1969) suggested that an inhibitor existed in the gelatinous matrix of *M.naasi* which suppressed egg hatch. However Ogunfowora and Evans (1976b) who washed *M.naasi* eggs thoroughly after their removal from the matrix material found no increase in hatching occurred. Meloidogyne infestations can persist in fallow soil or through periods of drought. Ogunfowora and Evans (1976a & b) found a proportion of the egg population failed to hatch despite favourable conditions and circumstantial evidence suggested these eggs may hatch the following season. De Guiran (1979) found 10- 20% of *M.incognita* eggs remained unembryonated during the normal development and hatching period, resuming development and emergence slowly over the following several weeks. This observation suggests the occurrence of an embryonic diapause, although no treatment has been successful in inducing these unembryonated eggs to develop synchronously (de Guiran (1979). An alternative suggestion put forward by Evans (1987) is the retention of genes which result in delayed embryonation in eggs due to selection pressures for survival. Populations in which juveniles undergo arrested development within the host (reviewed by Michel, 1974) during unfavourable conditions can be influenced by selection pressures suggesting the mechanism may have a genetic origin. This phenomenon is present in the nematode *Obelescoides cuniculi* (Watkins & Fernando, 1984). Continual selection for juveniles in a stage of arrest resulted in development of a population with a high percentage of arrested larvae. Removal of the selection pressures for arrested development, reversed the trend. Waller & Thomas (1975) found larvae of *H.contortus* populations in northern England were capable of arrest all year around, suggesting an adaption to the local climatic conditions. The termination of diapause does not always result in the resumption of hatching, favourable environmental conditions must also be present. The following nematodes, *M.naasi*, *H.avenae*, *H.rostochiensis* and *N.battus* all show post diapause quiescence if unfavourable conditions exist (Evans & Perry, 1976).

Host induced hatching among plant parasitic species has been most widely studied among the cyst nematodes and is reviewed by Shepherd (1962),

Shepherd & Clarke (1971), Okada (1975) and Clarke and Perry (1977). The dependence for hatching on the presence of root diffusates varies among the species. For example, *H.avenae*, *H.glycines* and *H.schachtii* although stimulated to hatch by the root exudates of various plants, all hatch freely in water. In *G.rostochiensis* substantial hatch only occurs when the cysts are stimulated by potato root diffusate (PRD); hatching is restricted in water. The active factor of the root diffusate which stimulated hatching of *G.rostochiensis* and *H.goettingiana* was found to persist in the soil for between 12 and 14 weeks after the host plants were removed (Perry *et al*, 1980 & 1981).

Artificial hatching agents have been found to cause markedly different responses in various species of *Globodera* (Clarke & Shepherd, 1971 and Clarke & Perry, 1981).

Attempts have been made to isolate and identify the natural hatching factor from various root diffusates including PRD (Perry, 1987) and kidney bean (Masamune *et al*, 1982; Fukuzaura *et al*, 1985). The isolation and structural determination of a natural hatching factor in the kidney bean was successful. In PRD, four to six hatching factors were present in small quantities and found to consist of organic acids able to act at extremely weak dilutions. Determination of the structure of hatching factors has important consequences concerning nematode control in the field, as it may allow the formation of analogues which can be used to induce hatching in the absence of host plants.

Studies on the hatching and exsheathment of infective stages of nematodes *in vivo* and *in vitro*, have been reviewed by Lackie (1975). A variety of combinations of the following physico-chemical factors were found to activate infective stages including CO₂-sodium bicarbonate buffer, pH, reducing agents, salts, temperature, enzymes and bile. The range of conditions employed in the various studies make it difficult to establish generalizations concerning hatching requirements. The majority of these studies involving ascarid eggs and trichostrongylid larvae of various species have shown that the same factors which influence moulting also influence hatching. This work has been extensively reviewed by Fairbairn (1960b), Rogers (1962, 1963), Rogers and Sommerville (1960, 1963 & 1968) and Sommerville and Rogers (1987).

Spontaneous hatching has been observed in a number of "infective" nematode eggs where it is more often termed background hatching. These include, *A.lumbricoides* (Rogers, 1960 and Fairbairn, 1961), *A.tetraptera* (Anyia, 1966) and *G.rostochiensis* (Ellenby and Perry, 1976).

Of infective stages studied most responded to CO₂ in the gas phase, and the concentration of dissolved gaseous CO₂ plus undissociated H₂CO₃. It has been proposed that the undissociated acid is the active agent in the stimulus of the CO₂-bicarbonate buffer (Rogers, 1979 & Petronijevic *et al*, 1986). Bicarbonate ions themselves do not act as a stimulus (Petronijevic *et al*, 1985). Reducing agents including cysteine, ascorbic acid, H₂S, SO₂ and sodium dithionite increased the activity of this stimulus with some species e.g. *A.suum*, *T.axei* (Hurley & Sommerville, 1982 & Rogers, 1960). These reducing agents were found to be most effective when the H₂CO₃ was low. Rogers (1960, 1980) and Petronijevic *et al* (1985) suggested that the efficiency of the stimulus was affected by the reducing agents rather than the substrates of the exsheathing and hatching fluids. It has also been suggested that they provide a stimulus by acting as weak acids (Petronijevic *et al*, 1985, 1986). Various infective stages which normally exsheath in the stomach of the host (*N.battus*, *N.dubius* and *H.contortus* exsheathed *in vitro* when stimulated by HCl (Christie & Charleston, 1965; Sommerville & Bailey, 1973 and Petronijevic *et al*, 1985). The presence of CO₂/H₂CO₃ in the medium often increased the effect of the stimulus. A selection of organic and inorganic acids was also found to stimulate exsheathment to varying degrees in the different species (Petronijevic *et al*, 1986). It was suggested that undissociated acid is absorbed, thus forming the active part of the stimulus. Undissociated bases were also found to stimulate ecdysis in *H.contortus* and *N.dubius* and hatching in *A.suum* (Petronijevic & Rogers, 1986).

The range of temperatures required for the hatching of "infective" eggs have been found to be closely related to the body temperature of the host, in both poikilotherms (Todd, 1944) and homoiotherms (Lackie, 1975).

The optimum temperature for hatching of ascarid eggs and exsheathment of trichostrongylid larvae has been found to be 40°C, with very little or no exsheathing and hatching occurring below 30°C (Fairbairn, 1961 and Rogers & Sommerville, 1968). However Rogers and Sommerville (1960) observed that

T. axei was "triggered" to exsheath in water at 38°C, following exposure to a suitable stimulus for 10 minutes at 38°C. No exsheathment occurred if juveniles were exposed to the stimulus for 10 minutes at 14°C, when subsequent incubation was at this temperature or 38°C. It was suggested that temperature was critical during some early phase, such as reception of the stimulus or closely associated processes.

Chitinase and several proteases, including pepsin, stimulated *D. viviparus* to exsheath *in vitro* (Silverman & Podger, 1964 and Parker & Croll, 1976). It was suggested that host enzymes may be responsible for exsheathing *in vivo*.

In summary, Sommerville and Rogers (1987) indicated that the major components of the host stimulus for the infective stages of nematodes appeared to be a temperature of 38°C, H₂CO₃ (pH 2-7) and, HCl (pH 2-3) to a lesser extent, both of which are absorbed rapidly in an undissociated form. It is suggested that CO₂ produced by the host acts as a stimulus by providing readily available H₂CO₃ from a dynamic equilibrium reaction involving CO₂ and H₂O over a wide range of pH values which allow infection to occur at various sites along the alimentary canal.

Rogers (1962) assumed that dissolved gaseous CO₂ and undissociated carbonic acid were required for the hatching of oxyurid eggs in combination with reducing agents, pH and temperature. However a review of the studies made on the hatching of oxyurids which parasitize homoiothermic animals suggest that oxyurids are less dependent on the host environment, requiring less specific environmental conditions than ascarids and trichostrongylids. The oxyurids, *O. equi* (Schwartz, 1923 and Gordon & Macfie, 1924), *E. vermicularis* (Deschiens, 1944 and Jones & Jacobs, 1941), *S. obvelata* (Deschiens, 1944 and Chan, 1952), *S. muris* (Philpot, 1924 and van der Gulden & van Aspert-van Erp, 1975) and *A. tetraptera* (Philpot, 1924; Deschiens, 1944; Hsu, 1951 and Anya, 1966) all hatched without stimulation by CO₂ and reducing agents. Hatching increased slightly in *S. muris* when stimulated by 5- 10% CO₂ and the reducing agents, bisulfite, cysteine and sodium dithionite. Anya (1966) found that 5% CO₂ only influenced hatching in *A. tetraptera* eggs in the absence of or the presence of very low concentrations of oxygen. The reducing agents sodium dithionite and cysteine had little effect on hatching.

Anya (1966) reported that the major factors which initiated hatching *in vitro*

in *A.tetraptera* eggs were a pH of 7.3–7.4, a temperature of 37– 40°C and an aerobic environment. In comparison with ascarid eggs (Fairbairn, 1961) he found no hatching occurred at temperatures below 30°C. Hsu (1951) obtained hatching between 20°C and 30°C. *S.muris* eggs were found to hatch at temperatures as low as 19°C (Philpot, 1924 and van der Gulden & van Aspert-van Erp, 1975), although in agreement with the other oxyurids, *E.vermicularis*, *S.obvelata* and *O.equi*, they hatched better at higher temperatures (Jones & Jacobs, 1941; Chan, 1952 and Gordon & Macfie, 1924). Bile was found to stimulate hatching in *S.muris* but inhibited hatching in *E.vermicularis*. Schwartz (1923) found *O.equi* emerged in varying numbers when eggs containing developed larvae were moistened with water or physiological saline.

The digestive enzymes pepsin and trypsin and, sequential treatment in acid followed by alkaline conditions were shown to stimulate hatching in several oxyurids. Immersion of *O.equi* eggs in an acid solution of pepsin did not cause hatching. However transfer of these eggs to pancreatic juice or saline solution of equivalent alkalinity, resulted in the emergence of active larvae (Gordon & Macfie, 1924). Hatching of *E.vermicularis* has also been reported in artificial gastric juice (Jones & Jacobs, 1941). Chan, 1952, who demonstrated that *S.obvelata* eggs which emerged in pepsin/HCl died very quickly found increased survival occurred when eggs were incubated in trypsin and sodium bicarbonate. Van der Gulden and van Aspert-van Erp (1975) found no hatching occurred in *S.muris* eggs incubated in pepsin/HCl, but large numbers hatched if eggs were incubated in trypsin and bile prior to incubation in buffer. *A.tetraptera* eggs were softened after incubation in pepsin/HCl and trypsin, but no emergence occurred (Philpot, 1924). Hsu (1951) found eggs hatched in artificial gastric juice followed by sodium bicarbonate.

From these various studies on oxyurid eggs it appears that they will hatch over a wide range of temperatures and pH values and in the absence of reducing agents and CO₂, suggesting they are less dependent on the environmental factors of the host gut (pCO₂, pH, redox potential and temperature) which are required to activate ascarid egg hatch and trichostrongylid larval exsheathment. Although nematode hatching is generally classified as two types, "spontaneous" and "infective" (Rogers, 1963), Bird (1968) considered the basic mechanism of hatching in each type to be similar.

The stimulus elicits a behavioural response in the larva which brings about increased larval activity, resulting in a breakdown of the lipid layer and an increase in plasticity of the eggshell and subsequent emergence.

The structure and function of the nematode eggshell has been reviewed by Wharton (1980a,b &1983) and described in Section 3.1. The majority of studies completed involve the development of the eggshell during oogenesis and/or identification of the layers present. Changes in the eggshell are essential for emergence to succeed, however few ultrastructural studies have been made of the nematode eggshell during the hatching process. From an ultrastructural study of the egg of *M.javanica* during hatching Bird (1968) observed that the lipid layer disappeared after active movement of the larva was initiated. Van Gulden and van Aspert-van Erp (1976) studied changes in the operculum prior to emergence in the oxyurid *S.muris* using scanning electron microscopy.

Unstimulated nematode eggs have been shown to be permeable to gases, water vapour and organic solvents, as discussed in Section 3.1. Various techniques have been used to show that further permeability changes occur in the egg prior to and during hatching. Increased plasmolysis of eggs of *A.duodenale* (Looss, 1911), *T.retortaeformis* (Wilson, 1958) and *A.tetraptera* (Anya, 1966) occurred shortly before hatching commenced. Van der Gulden and van Aspert-van Erp (1976) used the negative effect of peracetic acid on the hatching of *S.muris* eggs to demonstrate increased permeability during the initial stages of hatching. The penetration of various stains, dyes and fixatives into the eggs have been observed in *Ascaris* species (Fairbairn, 1961; Barrett, 1976 & Wharton, 1980), *N.battus* (Ash & Atkinson, 1984), *H.diesingi* (Wharton, 1980), *Ancylostoma* species (Matthews, 1986), *M.javanica* (Bird & McClure, 1976) and *G.rostochiensis* (Perry & Feli, 1986). Kaulenas and Fairbairn (1966) used radio isotope labelling to measure the development of permeability in *A.lumbricoides*. Measurements of trehalose in the hatching medium, indicated that trehalose leakage from eggs was initiated prior to emergence and subsequently increased during hatching of *A.lumbricoides* (Fairbairn, 1961), *N.battus* (Ash & Atkinson, 1984) and *G.rostochiensis* (Clarke pers. comm., cited Perry, 1987). An increase in the water content of larvae within the eggs after stimulation to hatch was found in *G.rostochiensis* (Ellenby & Perry, 1976) and *A.suum* (Clarke & Perry, 1980). An increase also occurred in the egg volumes of *M.javanica* (Wallace, 1966); *N.americanus* (Croll, 1974) and *C.elegans* (Croll,

1975) prior to eclosion. Both these changes demonstrate an increased permeability of the eggshells. Matthews (1985 & 1986) used time lapse video recording micro techniques to show the attainment of permeability during the development and eclosion of *Ancylostoma* species eggs.

Hatching mechanisms in cyst nematodes has been reviewed by Clarke and Perry (1977) and comparisons made between the hatching mechanisms of plant and animal parasitic nematodes by Perry and Clarke (1981). The hatching mechanisms for nematodes which hatch spontaneously have been examined in detail in *T.retortaeformis* (Wilson, 1958), *M.javanica* (Bird, 1968), *N.americanus* (Croll, 1974), *C.elegans* (Croll, 1975) and *Ancylostoma* species (Matthews, 1985 & 1986). In all these species, the attainment of activity by the larva is associated with a change in permeability of the eggshell to water shortly before hatching. Matthews (1986) found a second even earlier permeability change arose shortly after *Ancylostoma* larvae became active. Following these permeability changes various ideas on water uptake by the egg contents have been suggested in connection with emergence of the larva. Wilson (1958) found no increase in egg volume but he observed that movement of *T.retortaeformis* larvae became suppressed prior to hatching and considered that the eggshell burst by an increase in larval hydrostatic pressure. Wallace (1966) observed an increase in the eggsize of *M.javanica* prior to hatching and suggested it was due to water uptake by the larvae and considered that the increase in friction between the nematode and the eggshell was the stimulus for stylet activity to pierce the eggshell. Croll (1974 & 1975) reported a 15-20% increase in the egg volume of *N.americanus* and *C.elegans* and considered an influx of water into the egg fluid causing an increase in the internal pressure of the egg was responsible for rupture of the eggshell. Looss (1911) considered that the eggshell of *H.contortus* burst freeing the larvae. Matthews (1986) found no increase in the egg volume of *Ancylostoma* species prior to hatching. Bridges (1974) observed an increase in the size and flexibility of eggs of *Tylenchorhynchus maximus* prior to hatching. The larvae were observed to emerge by exertion of bodily movements against the shell rather than expulsion under pressure from the eggs. Perry & Clarke (1981) reported that osmotically increased internal pressure of the egg fluid appeared rarely to be involved with expulsion of larvae as the eggshell becomes less turgid once pierced by the stylet, as observed by Taylor & Whitlock (1962) with *A.avenae*.

The hatching mechanism of various "infective" nematodes described below have been examined in detail and share many similarities. The eggs of *Ascaris* species (Fairbairn & Passey, 1957 and Fairbairn, 1961), *N.battus* (Ash & Atkinson, 1984), *G.rostochiensis* (Clarke *et al*, 1978) and *H.goetingiana* (Perry *et al*, 1983) all contain trehalose in their eggfluid at concentrations varying between 0.1M and 0.5M and trehalose is leaked from the eggs when stimulated to hatch (Fairbairn, 1957 & 1961; Perry *et al*, 1983; Ash & Atkinson, 1984 and Clarke pers.comm; cited Perry, 1987). The effect of osmotic stress on the hatching of eggs, examined by investigating the inhibition of hatch at various concentrations and measuring the water content of larvae both free and unhatched have provided evidence for the following hatching mechanism. Prior to stimulation the eggshell of *G.rostochiensis* (Ellenby, 1974; Ellenby & Perry, 1976 and Clark *et al*, 1978) and *A.suum* (Clarke & Perry, 1980) act as a semipermeable membrane allowing the movement of water but not trehalose in both directions and the larvae are maintained in a quiescent state of incomplete hydration. Once hatching has been stimulated, the level of trehalose increases in the medium indicating a change in permeability of the eggshells from semipermeable to fully permeable. This loss of solute from the egg fluid allows hydration of the larvae to occur resulting in the initiation of larval activity and subsequent emergence.

Banyer and Fisher (1980) found *H.avenae* larvae freed mechanically from eggs were immotile in water until released from dormancy by exposure to low temperatures. They considered this physiological immotility to be distinct from the physical immotility found in *G.rostochiensis* due to the high osmotic pressure in the eggfluid (Clarke *et al*, 1978) They questioned the view that the trehalose concentration in the egg controls the water content of the larva. Instead they proposed that the rigidity of the eggshell was the factor limiting reduction of osmotic pressure and suggested an increase in egg volume of about 5% prior to hatching would allow motility to commence.

Van der Gulden and van Aspert-van Erp (1976) suggested that hatching of the oxyurid *S.muris* occurred as a result of increased water permeability of the eggshell. Investigations into the effects of temperature, cysteine and trypsin on the eggshell using the toxic effect of peracetic acid on hatching and SEM studies on the operculum, established that all three treatments increased permeability of the eggshell. They observed that motility of the larvae

increased at 37°C but not after stimulation by cysteine. It was considered therefore that Wilson's (1958) assumption that permeability increased as a result of emulsification of the lipid layer was unlikely to be the cause of permeability in *S.muris* eggs. Instead they favoured the release of enzymes as postulated by Rogers (1962). Observations on the hatching of eggs in paraffin oil suggested that water was necessary for opening of the operculum. They postulated 3 stages to the hatching mechanism:- stage 1, the eggshell became permeable to water as a result of host stimuli; stage 2, the larvae dissolved the chitinous seal in the presence of water and stage 3, uptake of water by the larva and emergence from the egg.

The measurement of the water content of the larvae of several nematode species using interference microscopy have been compared by Perry & Clarke, (1981). There is no significant change in the water content of *H.schachtii* (Perry, 1977a) and *N.battus* (Perry, 1977b) prior to hatching. The water content of unhatched *H.schachtii* in the egg is greater than that of *G.rostochiensis* larvae, even after *G.rostochiensis* larvae have taken up water prior to hatching. The difference in water content of the two species may reflect the lowered osmotic concentration present in the egg fluid of *H.schachtii* (Perry et al, 1980). This and the ability of *H.schachtii* larvae to remain motile in osmotic concentrations which are inhibitory to *G.rostochiensis* may explain their ability to hatch more freely in water (Perry et al, 1980). Larvae of *G.rostochiensis* (Ellenby, 1974), *H.schachtii* (Perry, 1977a), *N.battus* (Perry, 1977b) and *H.goetingiana* (Perry et al, 1980 and Beane & Perry, 1983) increased their water content after hatching, whereas the larvae of *M.incognita* (Ellenby, 1974) were fully hydrated prior to hatching. It is likely that the flexibility of the eggshell and space available determines the water content of the enclosed larvae, thus the 30% free space in eggs of *M.incognita* and the plasticity of the eggshell allows complete hydration of the larva prior to hatching. In contrast, the egg of *G.rostochiensis* is rigid and the larva completely fills the interior, therefore preventing complete hydration after stimulation to hatch. These variations may represent the differences in the life histories of the two nematodes (Ellenby, 1974). The flexibility of the eggshell and space availability for the larvae may influence the differences in hatching behaviour described in many nematodes and reviewed by Croll (1970), Croll and Matthews (1977) and Perry and Clarke (1981).

The observation that repeated short exposures to root diffusate elicited the same hatching response as continuous exposure to diffusate in eggs of *G.pallida* (Forrest & Perry, 1980) and *G.rostochiensis* (Perry & Beane, 1982) indicated that the enclosed larva was involved in the early stages of hatching. Behavioural studies by Doncaster and Shepherd (1967) indicate a delay in the response of the larva to the hatching factor

Indirect evidence for the involvement of enzymes in the permeability changes of the egg, especially the lipid layer have been found in many nematode species. It is unclear whether changes to the lipid membrane occur as a result of larval and/or enzymatic activity. Emulsification of the inner lipid layer of the eggshell of *T.retortaeformis* (Wilson, 1958) and *M.javanica* (Wallace, 1966 & 1968) was considered to result from both larval activity and the presence of emulsifying agents in the egg fluid. Bird (1968) from ultrastructural studies of the eggshell and larva indicated that enzymes synthesized in and secreted from the sub-ventral oesophageal glands prior to hatching caused hydrolysis of the lipid layer. Behavioural studies by Taylor (1962) and Flegg (1968) reported the occurrence of pumping movements in the anterior region of enclosed *A.avenae* and *Xiphenema diversicaudatum* larvae prior to hatching, which was followed by increased flexibility of the eggshell, implying the release of enzymes into the surrounding egg fluid. A change in plasticity of the eggshell, has been observed in several species including *M.javanica* (Bird, 1968), *T.maximus* (Bridge, 1974) and *N.americanus* (Croll, 1974).

Direct evidence of the involvement of enzymes in hatching has been demonstrated in *A.lumbricoides* (Rogers, 1958 & 1960; Fairbairn, 1961; Ward & Fairbairn, 1972 and Hinck & Ivey, 1976), *H.contortus* (Rogers & Brooks, 1977) and *H.glycines* (Tefft & Bone, 1985).

In *A.lumbricoides* the presence of a protease, lipase and chitinase were found in the hatching medium after release of the larvae (Rogers, 1958). As yet, secretion of these enzymes by the larvae has not been demonstrated. Barrett (1976) using molecular probes found no evidence of chemical or conformational changes to the ascaroside membrane and suggested that changes in the permeability of this layer were caused by mechanical damage

due to larval activity. In *H.glycines* (Tefft & Bone, 1985) and *H.contortus* (Rogers & Brooks, 1977), leucineaminopeptidase activity has been found in hatching fluid and egg homogenates.

The origin and location of enzymes involved in hatching is unclear. Clarke & Perry (1981) and Perry (1987) have recently presented the following concepts. The enzymes may not be secreted by the juvenile, but could be present in the eggfluid or eggshell. Activation may occur directly or indirectly, by the hatching factor (root diffusates) or removal of inhibitors (trehalose concentration). Enzymes in the egg fluid may be kept inactive by separation from their substrate by lipid membranes. In both these instances permeability changes of the lipid layer resulting in trehalose release from the egg fluid, precede enzymatic activity which erodes the outer egg layers of the eggshell. The possibility arises that enzymes which alter the permeability of the lipid layer may be located elsewhere in the eggshell or structural changes cause permeability changes in the lipid layer. In *H.schachtlii*, the lipid layer of the egg was only detected in eggs from cysts uncontaminated by fungi (Perry & Trett, 1986). It has been suggested that fungal enzymes such as lipase may enter the egg and disrupt this layer of the eggshell.

Ellenby & Gilbert (1957, 1958) observed that calcium ions had a synergistic effect on hatching and suggested that cation transport was involved in the hatching mechanism of *G.rostochiensis*. Further studies have shown that Ca^{2+} ions are a major inorganic constituent of the eggshell (Clarke, Cox and Shepherd, 1967). Using X-ray analysis, Atkinson and Ballantyne (1980) have shown that an uptake of Ca^{2+} ions occurs when eggs are exposed to the hatching factor, PRD. Atkinson and Ballantyne (1979) proposed that Ca^{2+} ions played an active role in the hatching process of *G.rostochiensis*. They found inhibition of hatching at low concentrations by specific inhibitors of calcium mediated processes, ruthenium red and lanthanum chloride and, enhancement of hatching by calcium ionophores which sequester calcium ions, allowing them to pass freely through membranes, thus initiating Ca^{2+} mediated processes. Atkinson and Taylor (1980) found that both ruthenium red and lanthanum chloride bound to eggs stimulated with PRD. The main site of ruthenium red binding was localised at the inner surface of the eggshell suggesting that a membrane contained a calcium binding glycoprotein (Taylor & Atkinson, 1980). It is proposed that Ca^{2+} ions are transported into the egg

by means of a sialoglycoprotein with a high affinity for Ca^{2+} ions (Atkinson & Taylor, 1983). A similar mechanism has been proposed for *N.battus* (Ash & Atkinson, 1984).

Clarke and Hennessy (1981) however, found that lanthanum chloride acted as a moderately effective hatching agent for *G.rostochiensis* with partial inhibition of PRD, which was probably due to complex formation. Evidence was presented indicating that ruthenium red inhibited hatching by inactivation of the hatching factor as opposed to interference with the hatching mechanism. As mentioned in Perry (1987), they considered that free Ca^{2+} ions were not essential for hatching and a calcium transport mechanism was not involved in the permeability change of the eggshell (Clarke & Hennessey, 1983, cited Perry, 1987). Recently Matthews (1986) has shown development and eclosion of *Ancylostoma* larvae were unaffected by ruthenium red and suggested that sieve like properties of the outer egg shell, most probably the chitin layer, had prevented it from reaching any calcium binding sites on the inner lipid layer, as proposed by Ash & Atkinson (1984). The smaller molecules of lanthanum chloride were shown to affect development rather than eclosion, thus the inhibitory effects of ruthenium. red and lanthanum chloride on hatching observed by Atkinson *et al* (1980) may be developmental in origin as opposed to affecting hatching.

Clarke and Perry (1985a & 1985b) have recently indicated that the role of Ca^{2+} ions in permeability changes during the hatching of *G.rostochiensis* and *H.schachtii* may be structural. X-ray analysis revealed three types of calcium binding site on eggshells of *G.rostochiensis*. Sites on the lipid layer appear to be involved in the hatching mechanism, as exposure of the eggshell to hatching agents result in them binding to or replacing Ca^{2+} ions. This may result in a change in the lipoprotein structure of the lipid membrane and increased permeability.

Petronijevic and Rogers (1986) considered calcium to be essential for the action of H_2CO_3 as the stimulus for ecdysis of infective *H.contortus* juveniles and drew parallels with the evidence for calcium involvement in hatching *G.rostochiensis* eggs.

Rogers, Sommerville and co-workers (reviewed in Rogers & Sommerville, 1963 & 1968 and Sommerville and Rogers, 1987) developed the hypothesis that

the stimulus from the host acted as a physiological trigger setting in motion a series of events in the larvae which were able to continue on withdrawal of the stimulus and terminated in hatching or exsheathment of the infective stages. They envisaged that the stimulus activated a receptor in the larva probably through some intermediary system which affected target cells and lead to the secretion of exsheathing and hatching enzymes. In *T. axei* (Sommerville, 1957 and Rogers & Sommerville, 1960) indicated that some region between the base of the oesophagus and nerve ring was involved in the reception of the stimulus and in storing and releasing exsheathing fluid. Neurosecretory cells and neurosecretory granules have been identified in *A. lumbricoides* larvae (Davey, 1964 and Rogers, 1968) in the vicinity of the nerve ring, however their function is unknown. Neurosecretory cells have been shown to be associated with secretory cells in *Phocanema decipiens* at the time of moulting (Davey and Sommerville, 1974. In addition Davey (1976), Davey & Goh (1984) and Goh & Davey (1984) have provided evidence that the secretion of exsheathing fluids by infective stages is under the control of the endocrine system.

Petronijevic and Rogers (1983) proposed a hypothesis for the induction of development of the parasitic stage from the infective stage which infect homoiothermic hosts. They suggested that the stimulus from the host (H_2CO_3 , pH, temperature) activated the endocrine system of the larvae which controlled ecdysis and hatching and the DNA transcription of the gene set of the following parasitic stage. Evans (1987) considers this hypothesis to be relevant to diapausing nematode eggs. He suggests that a block in development which occurs probably as a result of endogenous action or environmental stimuli, is removed when either further stimuli, perhaps from the host and/or surrounding environment, or a time delay, override the threshold level-required to maintain the eggs or larvae in a dormant state. The breakdown of inhibitors or the synthesis of hormones may be involved in the termination of dormancy. Juvenile hormone was found to maintain dormancy in *H. contortus* eggs, the effect was reversed by the hatching stimulus. A similar hormone has been found in the infective stage of *H. contortus* suggesting that it may act by controlling dormancy in the fully developed egg until hatching stimuli are applied (Rogers, 1979 & 1980).

Rogers and Sommerville (1987) have proposed a sequence of events linking

the stimulus for development with ecdysis and hatching in nematodes. They suggested that the host stimuli resulted in the production of nor adrenaline by the larvae (Rogers and Head, 1972), causing the release of neurosecretion which activated leucine aminopeptidase in the excretory cell to breakdown cellular membranes of cells containing the ecdysial and hatching enzymes (Rogers, 1982).

4.2 Hatching *in vitro* and *in vivo*

4.2.1 Introduction

This chapter examines the hatching of *A.tetraptera* eggs *in vitro* when exposed to a variety of physico-chemical factors either singularly, combined or sequentially. These factors include temperature, pH, osmotic pressure, HCl, enzymes-pepsin and trypsin, bile salts and reducing agents, such as cysteine, sodium dithionite and dithiothreitol. The site of hatching *in vivo* is also examined.

Several authors, Philpot (1924), Hsu (1951) and in particular Anya (1966) have previously investigated aspects of hatching *in vitro* and their findings are described in the literature review below. However difficulties encountered in obtaining levels and rates of hatch using a method devised by Anya (1966) for optimum hatching (mentioned previously in Section 3.2.1.), led to a reinvestigation of some of the factors which he had studied, that is pH, temperature, reducing agents and osmotic concentration. Although modification of his method improved the level of hatch obtained, the rate of hatch was still much reduced. Therefore a preliminary investigation of *in vivo* hatching was undertaken to establish the site of hatching in the host gut. This provided further indications of physico-chemical factors which may stimulate hatching *in vitro*, and allowed a comparison with the results obtained by previous authors.

Philpot (1924), Chan (1955), Anya (1966b) and Behnke (1974) have previously studied aspects of the lifecycle of *A.tetraptera in vivo*, as reviewed in Section 3.1. The majority of these studies were concerned with the pattern of migration and the development of larvae within the host and their distribution along the mouse colon. Therefore most of the initial observations were made either 12 or 24 hours post infection, by which time the process of hatching had been completed. However Philpot (1924) and Anya (1966b) in particular observed hatching in the host. In a study of *in vivo* hatching, Philpot (1924) obtained free larvae from the posterior end of the small intestine and

caecum of mice four hours after feeding. No figures on dosage levels, larval recovery or state of hatching are given. Anya (1966b) observed that no larvae were recovered from either the stomach or the upper intestine suggesting no hatching had taken place in these regions. Larvae were recovered only from the lower intestine or caecum in the first 5 hours and in the 6th hour, very few larvae (5) were recovered from the colon. Anya (1966) concluded that eggs hatched in the intestine and caecum of the host, usually within the first two hours, although it's initiation occurred earlier. 114

The establishment of the site of hatching *in vivo* led to the reinvestigation of factors briefly studied by Philpot (1924) and Hsu (1951). Hydrochloric acid, pepsin/HCl and trypsin were examined at different concentrations and periods of incubation to try and increase the rate of larval emergence. The same investigative routine was also used to examine the reducing agent dithiothreitol, which stimulated hatching unlike the reducing agents, sodium dithionite and cysteine studied by Anya (1966).

Anya (1966) employed uterine eggs in his series of experiments, however faecal eggs were used throughout this present study. The results were deemed comparable with Anya's because experiments described in Section 3.2. & 3.3. had indicated that no differences existed between the two types of egg in either larval development or egg structure.

Literature review

The environmental conditions present in the mammalian gut in relation to the host-helminth interaction have been reviewed extensively; Hobson (1948), Crompton (1973), Mettrick & Podesta (1974), Lackie (1975), Barrett (1981) and Smyth (1976). Literature describing the digestive system of mice is reviewed by Foster *et al* (1983) and briefly summarized below. The alimentary canal of the mouse consists of the oesophagus, stomach, small intestine (divided into the duodenum, jejunum, and ileum), and the large intestine which consists of the blind-ending caecum, colon and rectum. The oesophagus is a short straight tube extending from the pharynx to the stomach. The stomach contains a non- glandular (cardiac) and glandular (pyloric) region. It opens into the small intestine via the pyloric sphincter which prevents the return of duodenal contents to the stomach. The small intestine consists of a coiled tube, recognised histologically as three separate regions, the duodenum, jejunum and ileum which opens into the caecum an elongated sac. There is no appendix in the mouse and the large intestine continues as the colon and terminates at the anus via the rectum. ^a

The gut is the largest endocrine gland in the body. In the stomach, parietal cells secrete intrinsic factor and HCl, chief cells secrete pepsinogen and cells in the neck of the gastric gland secrete mucus. HCl helps to break up protein bonds and activates some enzymes secreted by the stomach. Pepsinogen is activated autocatalytically to form the active pepsin, at a pH below 6. Pepsin attacks inner protein linkages to free carboxylic groups and form large peptide molecules. The mouse secretes gastric juice continuously, between 0.8 and 1.8ml in 3 to 6 hours, with a resulting pH of 2.5 (Ogawa and Necheles, 1958). Enzymes known to occur in the stomach tissue of the rat, but not confirmed in the mouse include carbonic anhydrase, lipase, pepsin and urease (Spector, 1956). ¹²

Pancreatic juice is a weakly alkaline, colourless, isotonic, bicarbonate rich solution containing many of the digestive enzymes which pass into the small intestine via the pancreatic duct. There is a high basal secretion of enzymes in the mouse pancreas (Danielsson, 1974) including amylase which hydrolyses sugars, lipase which hydrolyses glycerol and fatty acids emulsified by bile and colipase which is necessary for lipase to be effective in the duodenum.

Amylase, lipase, trypsin and carbonic anhydrase are present in rat pancreatic tissue (Spector, 1956). Trypsin, secreted as trypsinogen and activated by enterokinase requires a slightly alkaline pH (7-9) to function. It hydrolyses peptide bonds adjacent to a basic amino acid which carries two amino groups (lysine or arginine).

Bile is synthesized by the liver cells and passes into the duodenum via the bile duct. Bile acids emulsify fatty acids which are then kept in solution with the aid of bile salts. Major components of the bile fraction in mice include chenodeoxycholic acid and cholic acid (Beher *et al*, 1963). Alpha- and B-miricholic acids (Florkin and Mason , 1962) and 7-hydroxycholesterol (Danielsson, 1961) have also been found in mouse bile.

Although the small intestine does not secrete digestive enzymes, there is considerable digestive activity in its lumen or on its surface from proteolytic, lipolytic and amylolytic enzymes secreted by the pancreas. In the small intestine, intestinal juice, a viscous mucus is secreted by Brunner's glands and cells lining the crypts of Lieberkuhn. Brunner's glands in the mouse have both serous and mucous cells (Friend, 1964). Spontaneous secretion of Brunner's gland occurs and feeding increases secretion (Florey and Harding, 1934). Cizek (1954) found the gut contents and gut water made up between 7.5 and 9.5% and between 6 and 8% respectively of the body weight.

The main function of the caecum is water absorption from the intestinal contents. It also provides a site for further digestion of food by pancreatic enzymes and bacterial and protozoan microflora. The large intestine lacks digestive enzymes, but in the mouse it can actively absorb sugars and amino acids (Madge, 1975). The proximal portion absorbs sodium and chloride and the products synthesized by bacteria and secretes potassium and bicarbonate. The distal portion, contains undigested food remains and a large population of bacteria and stores and controls the expulsion of faeces.

Substantially more literature is available on conditions occurring in the gastro intestinal tract of the rat and is presented below as an indication of the physico-chemical environment present in a rodent gut.

In rodents the osmolality and concentration of the major ions, Na^{2+} , K^{+} , Ca^{2+} , Cl^{-} and HCO_3 in the fluid of the small intestine tend towards that of

blood plasma. Following feeding the osmolality of the small intestine rises from 210 to 290mOsm/L. Colonic NaCl concentrations are much lower, K^+ ions much higher and HCO_3 concentrations approximately the same as in blood plasma. The osmolality of the fluid is slightly hypertonic to that of plasma (Fordtran and Locklear, 1966).

Calloway (1968) calculated the proportions of the following gases, oxygen, carbon dioxide, hydrogen and methane present in the lumen of the stomach and intestine. He found the percentage by volume for methane was low, 0-5% in the stomach. Oxygen decreased in the stomach from 11-12% to 3-7% after eating. In the intestine it rose slightly during digestion from 1-8% to 2-10% and then decreased to 1-4%. Carbon dioxide rose from 13-18% to 16-34% in the stomach after feeding and then decreased to 14-19%. In the caecum it reduced slightly from 18-72% to 25-47% and then increased to its original range. Hydrogen decreased in the stomach from 14% to 1.5% and in the caecum from 16-33% to 1-16% after feeding.

Ogawa and Necheles (1958), in the rat and Haiba (1954), in the mouse, obtained pH values of 2.5 and between 3 and 6.5 respectively for the contents of the stomach. The pH of the intestinal lumen of the rodent is rarely alkaline (Calloway, 1968 and Levine, 1971). Haiba (1954) measured the pH of the intestinal wall and contents *in situ* using glass electrodes. He measured a pH of between 6 and 7.5, with a trend towards alkalinity in the posterior region of the alimentary tract, especially in the wall. The caecum was found to be acid again with a pH value of between 6 and 6.5.

The intestinal lumen has strong reducing tendencies which also decrease along a gradient down the intestine, with an Eh of -100mV in the proximal gut, decreasing to -200mV in the terminal ileum, colon and caecum (Bergiem *et al*, 1945).

Several authors have previously examined *in vitro* hatching of *A.tetraptera* eggs (Philpot, 1924; Deschiens, 1944; Hsu, 1951 and Anya, 1966). Philpot (1924) in the primary study initially observed the effect of temperature, enzymes, acid and alkaline on hatching. She found pepsin and trypsin at 37°C, softened the shell allowing embryos to be easily pressed out under a coverslip, although emergence was not spontaneous. Emergence was induced in sodium bicarbonate solution, although the larvae rapidly died. Embryos were also

freed by stirring the culture and allowing it to dry and remoisten, no movement of the larvae was detected in water.

Deschiens (1944) observed that larvae were freed from eggs incubated in water at 37–40°C for 5–6 hours. Hsu (1951) tested the effect of a range of solutions of various dilutions at temperatures of 28°C and 37°C for 5–6 hours on egg hatching. He found no hatching or low hatching occurred in solutions of higher concentration, at 37°C, or in solutions of any dilution at 28°C. At 37°C, a level of 65% hatch was attained in 50% or 25% artificial gastric juice after 4.5 hours and 17 hours, respectively. An equivalent hatch was achieved in 0.5% Na₂HCO₃ solution after 25 hours. Hsu (1951) found eggs hatched after incubation in artificial gastric juice followed by sodium bicarbonate solution however no results were presented. Low levels or no hatching occurred in human sweat, urine, saline, horse serum, albumin solution, HCl, water, sugar and Locke's solution.

In the most comprehensive study completed, Anya (1966), examined the role of temperature, pH, oxygen, carbon dioxide, reducing agents and osmotic concentrations on the hatching of *A.tetraptera* eggs previously incubated in distilled water for 9–10 days at 24°C. The number of hatched larvae was counted as the number of free larvae in the incubating medium plus the number of eggs which possessed a notch in the hardshell at the end of the experimental period (8 hours). In an attempt to control background hatching (those eggs which have hatched prior to exposure to the hatching stimulus), eggs were washed by centrifugation with the buffer solution at the pH of subsequent incubation. Eggs which showed no evidence of hatching were used for the following experiments. Controls showed background hatching to vary between 2 & 8%. Anya (1966) found major factors in the initiation of hatching were pH (7.3– 7.4), a temperature of 37–40°C, and an aerobic environment. He found hatching was much reduced under acid conditions (pH 6 or less) and alkaline conditions (pH9 or more). Temperatures of either less than 30°C or more than 45°C and low oxygen tensions resulted in low levels of emergence. He found CO₂ only influenced hatching in the absence or presence of low concentrations of oxygen. Reducing agents had little or no effect on hatching and 0.1–1M concentrations of NaCl and sucrose, in 0.1M buffer and physiologically balanced saline with increasing molar concentrations of NaCl were inhibitory to hatching.

4.2.2 Procedures and Results

Hatching in vivo

Materials and methods




After 9 to 10 days incubation at 25°C eggs were transferred to a small conical flask and adjusted to obtain the required dose (800 eggs in a 0.1ml suspension). A magnetic stirrer was used to ensure an even egg distribution in the suspension and egg counts on sample doses showed that the number of eggs varied by no more than 10%. The inoculum was administered orally to 7 infection free CBA/Ca/T6 mice via a stomach tube. At intervals of 0.25, 0.5, 1, 2, 4, 8 and 12 hours, 1 mouse was removed from the group and killed by means of CO₂ gassing. Immediately after death the alimentary canal was removed and divided into the following sections:- oesophagus, stomach, small intestine, caecum and colon. The small intestine was further subdivided into 3 equal sections. Each section was placed in a labelled bijoux bottle and frozen at -18°C until examination. On thawing, each section was opened under water in a separate grid marked petri dish and scraped clean in order to dislodge any larvae or eggs present on the gut wall. The number of unhatched eggs, empty eggshells, partially and fully emerged larvae present was counted under a binocular microscope. The pieces of gut were further examined for the presence of larvae on the mucosal wall by the slide compression technique. The control, a mouse inoculated with a 0.1ml egg free suspension was killed at the end of the experiment and its gut examined for the presence of any *A.tetraptera* stages. Faecal matter present in the large intestine was also examined by the salt flotation technique described in Section 2.2 for the presence of eggs and/or larval stages. The results are presented in Fig.6.

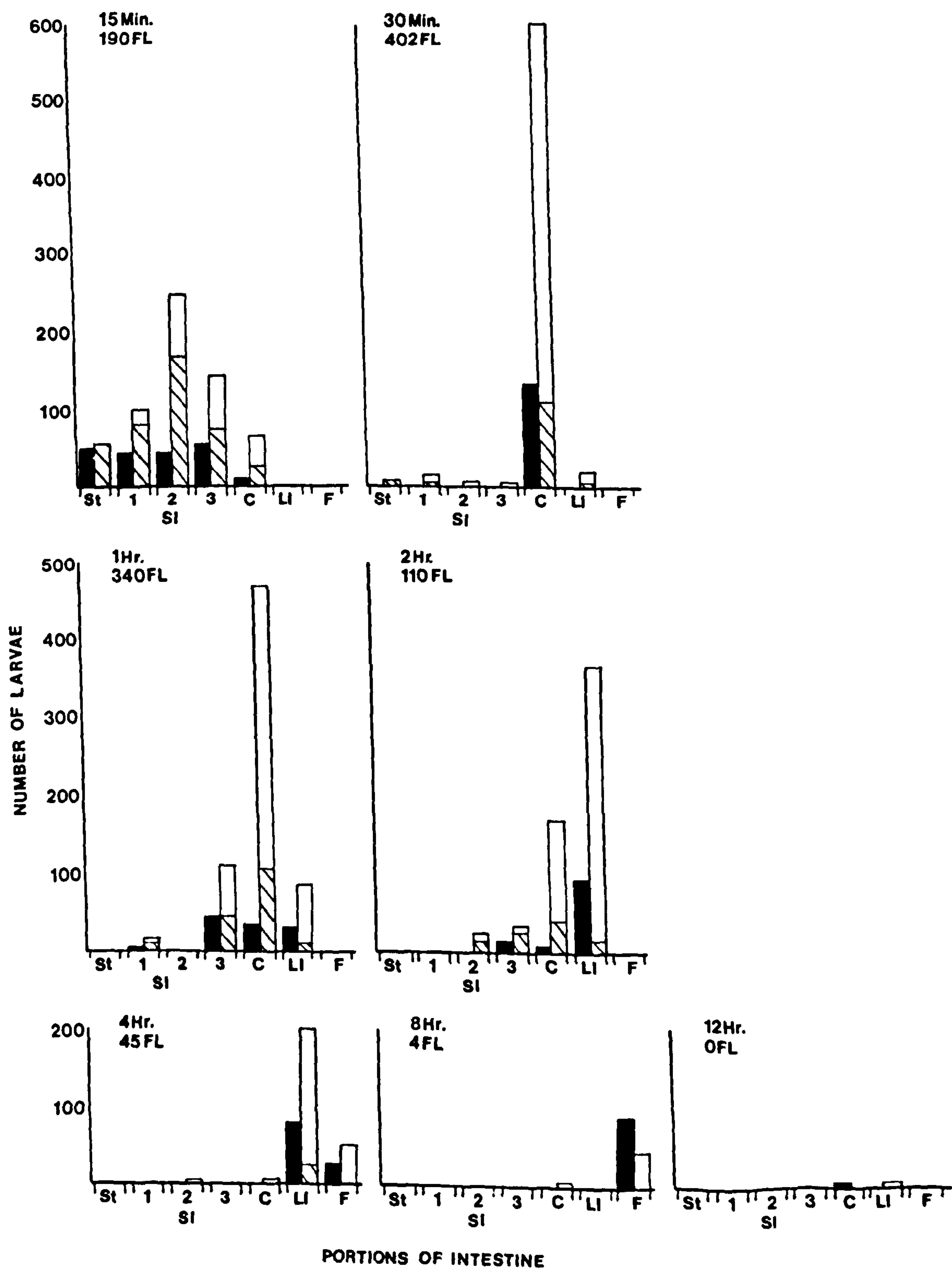
Results

Fig.6. shows the number of hatched and unhatched eggs recovered from different regions of the alimentary canal of infected mice over a period of 8 hours. The number of free larvae recovered is also recorded.

Eggs were present in faecal matter in the rectum 8 hours post infection. Unhatched eggs and hatching eggs, both partially emerged larvae and empty eggshells were found along the length of the gut from the stomach to the

Fig.6.Hatching *in vivo*

The number of unhatched eggs (), partially hatched eggs () and empty eggshells () recovered from the following sections of the mouse gut:- stomach (St), smallintestine (St:1, 2, 3), caecum (C), large Intestine (LI) and faeces (F) at regular intervals over a period of 12 hours. The number of free larvae (FL) found is also given. For each infection, 800±80 eggs were administered.



caecum, 15 minutes post inoculum. Only unhatched eggs and partially emerged larvae were found in the stomach. A higher proportion of partially emerged larvae were found in the upper small intestine, whereas empty eggshells were more in evidence in the lower small intestine and caecum. The majority of eggs recovered after 30 minutes and 1 hour post infection were found in the latter portion of the small intestine, the caecum and the colon. The bulk of these eggs consisted of empty eggshells particularly in the caecum. The proportion of empty eggshells present in the colon increased in mice killed 2 and 4 hour post infection. The recovery of fully emerged larvae decreased drastically 2 hours post inoculum. Unhatched eggs and a reduced number of empty eggshells were recovered from faecal matter 8 hours after infection. No evidence of infection was found in the control.

The effect of various physico-chemical factors on the hatching of *A.tetraptera* eggs.

For all the experiments described below eggs were collected and extracted from faecal sources as detailed in Section 2.2. Unless mentioned eggs were incubated as described in Section 2.3 for 9-10 days at 25°C in order to allow comparisons to be made with experiments performed by Anya (1966). The number of larvae which partially and/or fully emerged was estimated as described in Section 2.4. Background hatching was estimated as described in Section 3.2.2. and was found to be no greater than 5% in any of the incubation media examined at 25°C.

pH

Materials and Methods

A selection of buffered solutions pH 2-12 were prepared from the following 0.1M buffers, acid phosphate (pH 2), phosphate (7, 11), Tris (pH 7, 9), veronal acetate (pH 7, 9) (Pearse 1960); citrate (pH 4), borate (pH 9) (Clark 1928) as used by Anya (1966) and denoted "Anya's" buffers in future references. Biological buffers, Mes (pH 6.5), Pipes (pH 7.5), Tris (pH 7.5, 8.5), Tes (pH 7.5), Hepes (pH 7.5), Bicine (pH 8.5), Ches (pH 9.5) and Caps (pH 10.5) (Good *et al*, 1966, Sigma Ltd.) were prepared at 0.05M concentration. Four replicates (approx. 50 eggs each) were incubated at each of the pH's of the above buffers in 4-well multidishes (Gibco Ltd.) at 37°C overnight and examined for

evidence of hatching. The results are presented in Figs.7a & b.

Results

Figs.7a & b show the mean \pm SE percentage hatch obtained for eggs after 7 & 20 hours incubation at 37°C in a selection of buffers (pH range 2-11) including those used by Anya (1966) and biological buffers (Good, 1966). Data obtained by Anya (1966) under equivalent conditions is also presented.

Maximum levels of hatch for both the biological and "Anya's" buffers were found to occur in the pH range 6-10. Eggs incubated in biological buffers (pH 6-10) for 20 hours at 37°C, attained maximum levels of hatch of between approx. 75% and 90% (Fig.7b). The highest percentage hatch (90 \pm 5.5%) was obtained from eggs incubated in Bicine buffer (pH 8.5). The maximum number of larvae which fully emerged was highest for the biological buffers Pipes (pH 7.5), Bicine (pH 8.5) and Tris (pH 9.5), reaching levels of 65 \pm 4.5%, 80 \pm 3% and 72 \pm 3% respectively. Eggs incubated in "Anya's" buffers (pH 6-10) in comparison attained much lower maximum levels of hatch and full emergence of approx. 50-60% and 15-25% respectively (Fig.7a).

The levels of hatch obtained in both biological and "Anya's" buffers after 7 hours incubation at 37°C were between approx. 10 and 20% and 2.5 and 15% respectively. Considerable differences were found between these data and the levels of hatch reported by Anya (1966) after 7 hours incubation. These differences are examined in the discussion.

Temperature





Materials and Methods




For each of the following temperatures, 21°C, 25°C, 31°C, 37°C, 45°C and 50°C, 3 replicates (approx. 100 eggs each) were incubated in 0.05M Bicine Buffer (pH 8.5) in a 4-well multidish and examined at periodic intervals over 24 hours for evidence of larval emergence. The results are presented in Figs.8a & b.

Results

Fig.8a shows the effect of temperature on mean \pm SE percentage hatch, plus

Fig.7.The effect of pH and buffer on hatching of eggs

a) Mean \pm SE percentage hatch obtained in a selection of the following 0.1M buffers, pH 2-11:- acetate(a), citrate (c), borate (b), phosphate (p) and tris (t). at 37°C by Anya (1966) () and in the present study () after 7 hours incubation. The number of larvae which partially () and fully () after 24 hours is also illustrated.

b) Mean \pm SE percentage hatch obtained in a selection of biological buffers, Mes, Bicine, Tris, Ches and Caps, pH6.5-10.5, at 37°C, after 7 hours incubation (). The number of partially () and fully () ^{emerged} larvae after 24 hours incubation at 37°C is also given. For each value, n=4 replicates of 50 eggs each.

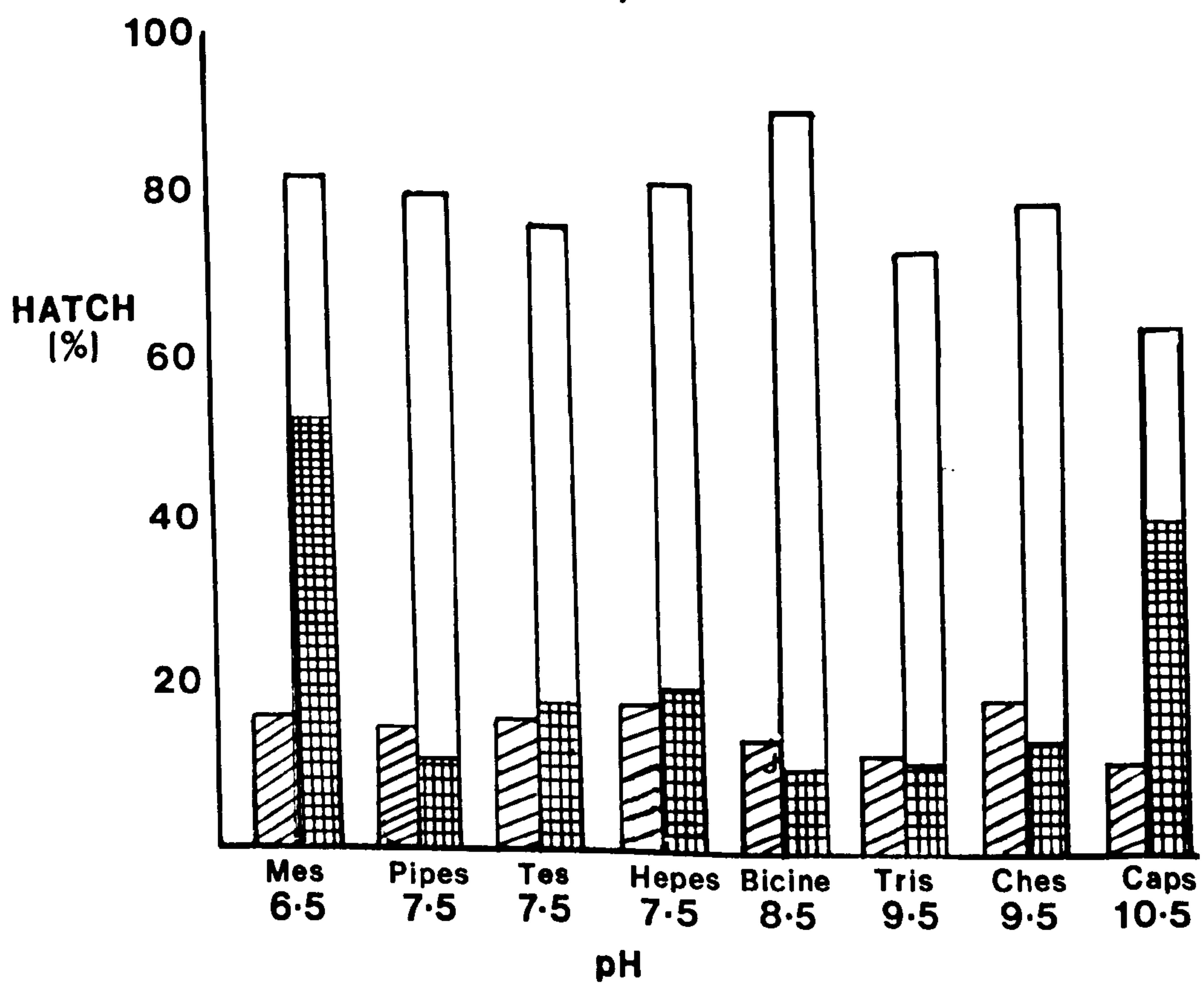
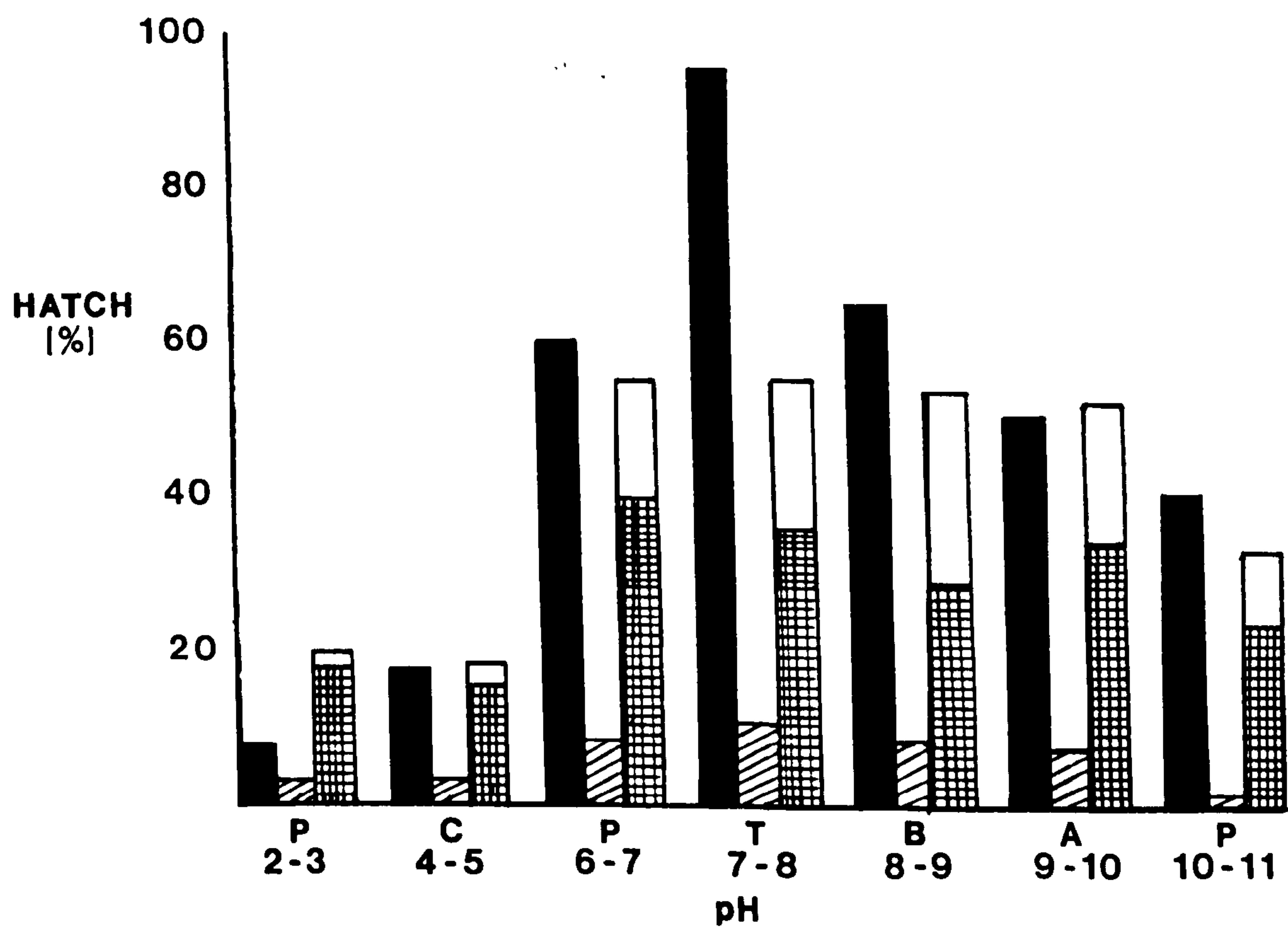
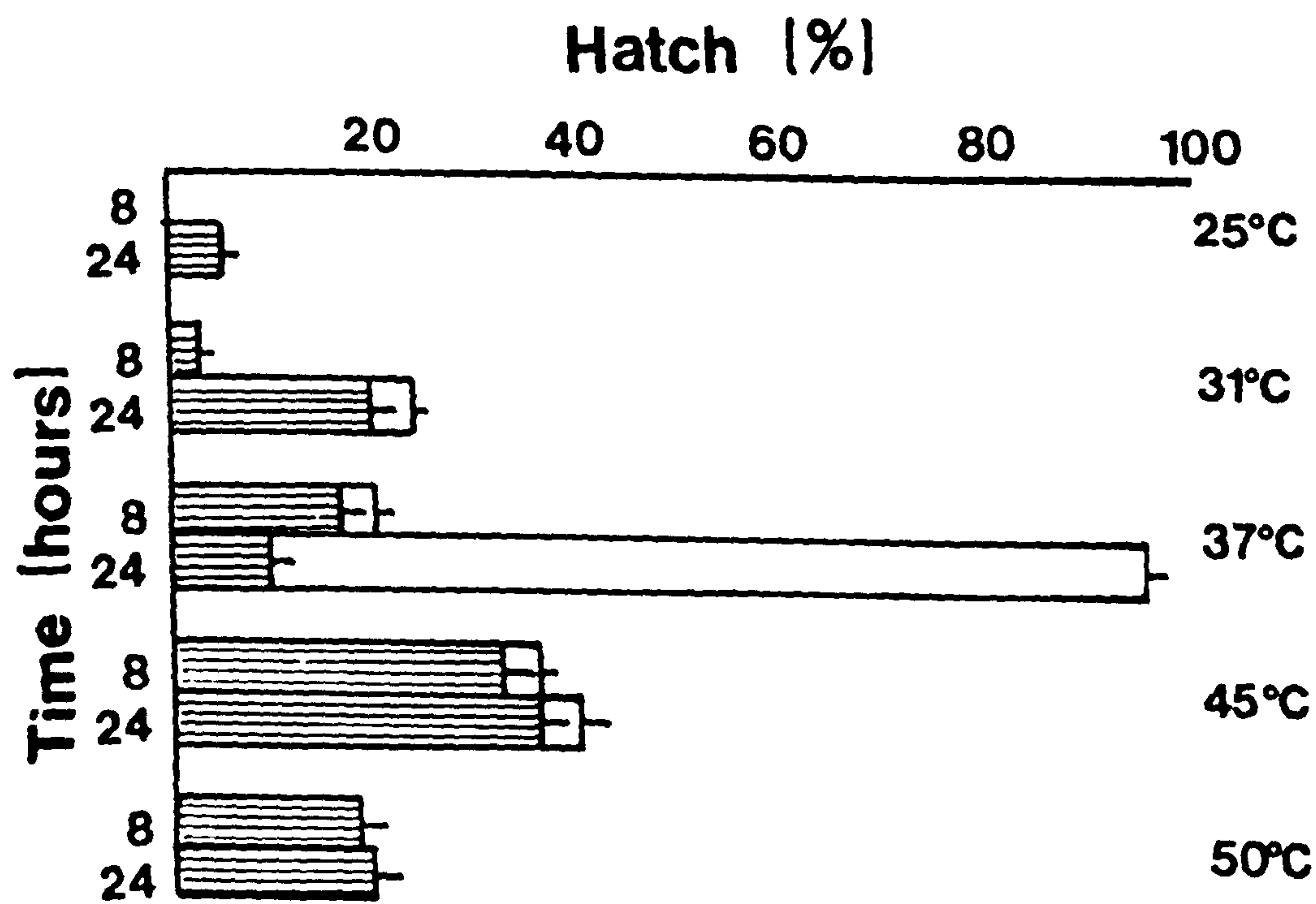
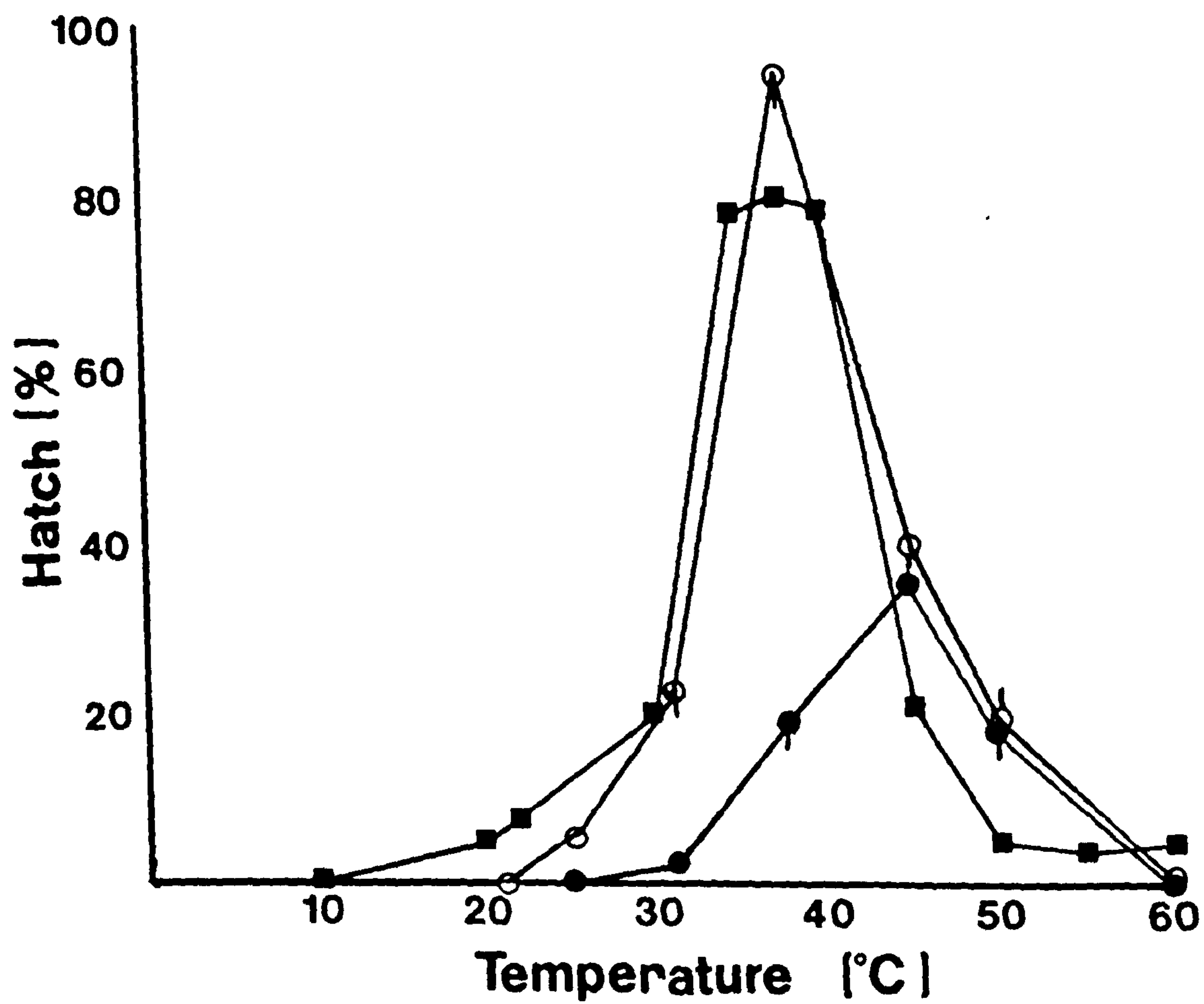


Fig.8.The effect of temperature on hatching.

a) Compares:- i) Mean \pm SE percentage hatch obtained at a range of temperatures (10° – 60° C), after 8 hours (●—●) and 24 hours (○—○) incubation in 0.05M Bicline buffer (pH 8.5) with, ii) the percentage hatch obtained by Anya (1966) after 8 hours incubation in a variety of 0.1M buffer solutions (■ ■) at equivalent temperatures.

b) Mean \pm SE percentage of larvae which partially (▤) and fully (□) emerged in the present study. For each value, n=3 replicates of 100 eggs each.



comparable data from Anya (1966). The levels of hatch obtained increased with temperature, up to 37°C, but decreased with further increases in temperature. After 8 hours incubation, maximum levels of hatch had occurred at 45°C and 50°C and were 36±2% and 18±2.5% respectively. The mean± SE percentage hatch obtained at 31°C and 37°C after 8 hours was 2.5± 0.5% and 19.5±1.5% respectively, no emergence occurred at 25°C. After 20 hours incubation the levels of hatch obtained for 25°C, 31°C, 37°C, 45°C and 50°C were 5.5±1%, 23.5±2%, 94.5±1%, 36±2% and 18±2.5% respectively. The differences between the present data and Anya's results (Anya, 1966) are examined in the discussion. Fig.8b shows the percentage of juveniles which have partially or fully emerged from the egg after 8 and 20 hours incubation at each temperature examined. After 8 hours incubation the majority of larvae which hatched at any of the following temperatures:- 31°C, 37°C, 45°C and 50°C were only partially emerged. At the final observation, after 20 hours incubation, the increase in total hatch which had occurred at all temperatures consisted mainly of partially emerged larvae excepting those incubated at 37°C where 85.5±3% of the larvae had fully emerged.

The effect of length of incubation at 37°C on further hatching of eggs when incubated at 21°C.




Materials and methods

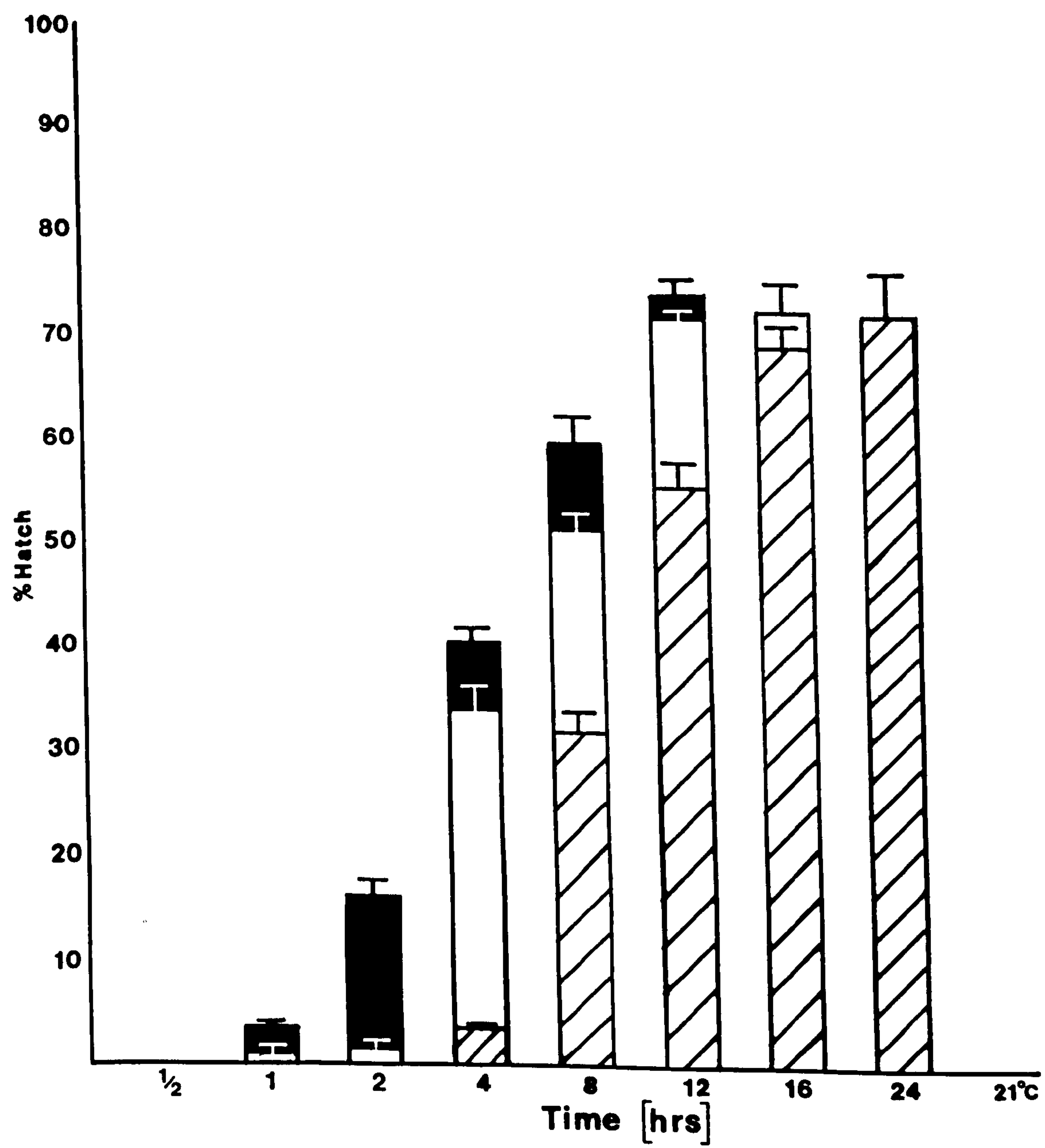
Eggs were incubated at 21°C for 5 days. Ten replicates of 30 to 50 eggs each were incubated at 37°C in 0.05M Bicine buffer (pH 8.5) for varying periods over 24 hours prior to incubation at 21°C for a further 48 hours. Hatching was estimated and the results are given in Fig.9.

Results

The mean±SE percentage hatch for each period of incubation at 37°C, plus further hatching at 21°C after 24 and 48 hours is given in Fig.9. Hatching occurred at 37°C when eggs were incubated for between 4 and 24 hours. Increased incubation at 37°C gave increased hatching with a maximum of 73±4% after 24 hours incubation. For all periods of incubation at 37°C, excepting 0.5 and 24 hours further hatching occurred when eggs were incubated at 21°C for 48 hours.

Fig.9.The effect of length of incubation at 37°C on further hatching at 21°C.

The mean \pm SE percentage hatch obtained from eggs incubated in 0.05M Bicine buffer (pH 8.5) at 37°C for different lengths of time () and at 21°C., following incubation of eggs for 24 hours () and 48 hours (). For each value, n=10 replicates of 30- 50 eggs each.



The longer the period of incubation at 37°C, the higher the proportion of remaining viable eggs which hatched at 21°C within 24 hours. For eggs incubated at 37°C for between 4 and 16 hours, the majority of hatching at 21°C occurred within 24 hours. No eggs hatched when incubated at 21°C alone. For eggs incubated at 37°C for 1 and 2 hours, all hatching occurred following incubation at 21°C, the majority between 24 and 48 hours. Eggs previously incubated for 12 and 16 hours at 37°C obtained a maximum hatch of between about 70 and 75% after incubation at 21°C. Eggs incubated at 37°C for between 1 and 8 hours progressively increased the total hatch obtained after incubation at 21°C.

Osmotic concentration



Materials and Methods

Molar concentrations of 0.5M, 0.25M, 0.125M, 0.06M and 0.03M NaCl and sucrose were made up in a 0.05M Bicine buffer (pH 8.5) and in water. Equivalent concentrations of Bicine buffer (pH 8.5) were also prepared. Five replicates of 30-70 eggs each were incubated in each of the molar concentrations of the five solutions:- NaCl in buffer, sucrose in buffer, NaCl in water, sucrose in water and buffer only in Nunc microwell '96 plates (Gibco Ltd.). The eggs were examined periodically for hatching over a 24 hour period. The osmotic concentrations of the various solutions were measured using a Precision Research Osmometer (Advanced Instruments inc., 1000 Highland Avenue/617-449-3000, Needham Hts, Massachusetts 02194). The results are presented in Fig.10 and Table 7.

Results

In all solutions, increasing osmotic concentration caused decreased hatching of eggs. The highest number of larvae which fully emerged occurred in osmotic concentrations of 137.5 mOsm/kg or less. Eggs incubated in sodium chloride and sucrose concentrations made up in buffer solutions produced a higher maximum hatch and percentage full emergence than eggs incubated in solutions of NaCl and sucrose in water at equivalent osmotic concentrations, Fig.10a/c & b/d. The maximum total hatch, between about 95% and 98% and the highest percentage full emergence, between about 90% and 95% were obtained in a 0.03M and 0.06M Bicine buffer with an osmotic

Fig.10.The effect of osmotic concentration on egg hatching.

The mean \pm SE percentage of larvae which partially () and fully () emerged, following incubation in a range of molar concentrations (0.03-0.5M) for 24 hours at 37°C. For each value, n=5 replicates of 30-70 eggs each.

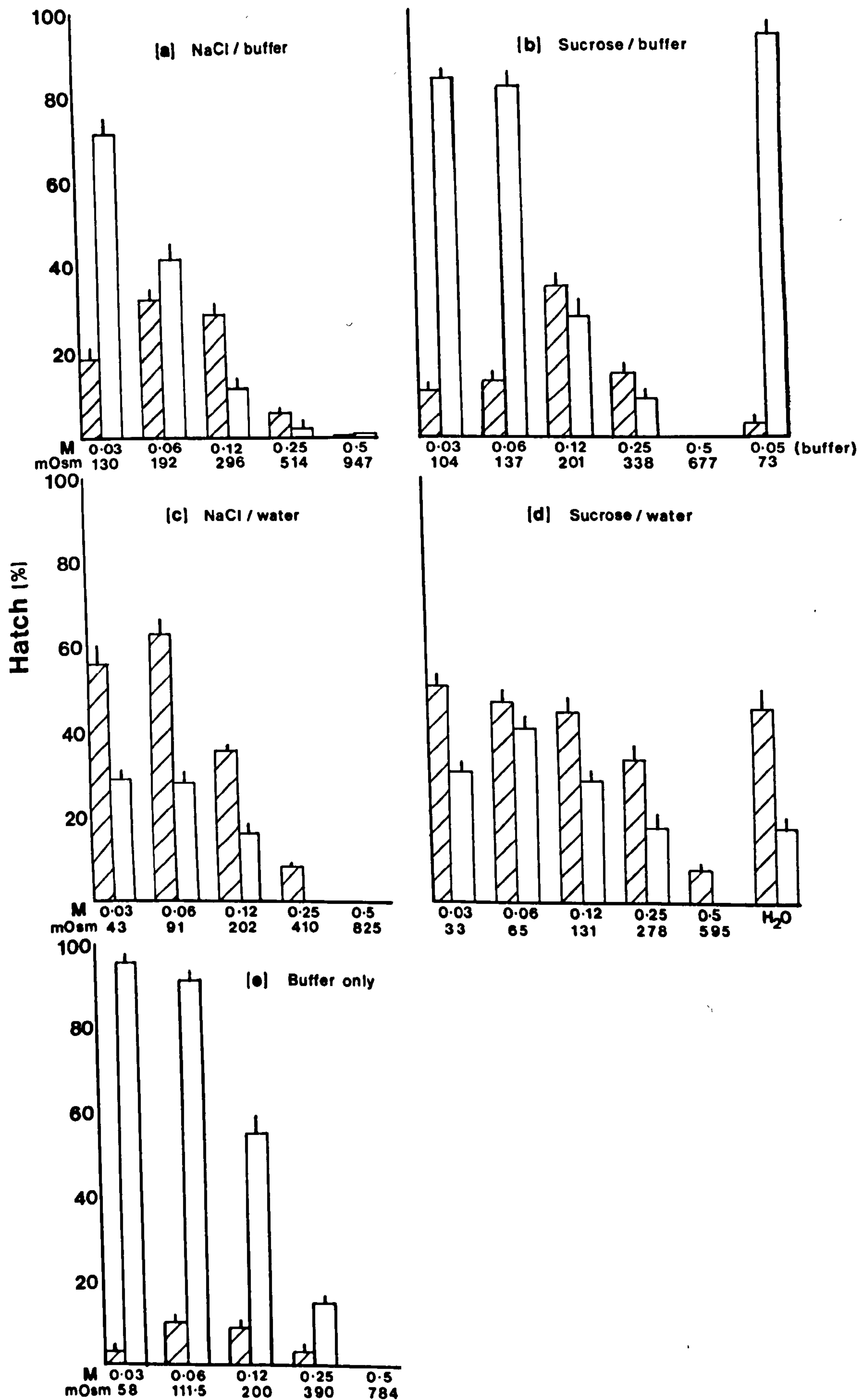


TABLE 7

The data presented below gives:- i) the time taken for eggs incubated in various treatments at a range of molar concentrations, at 37°C to reach 50% hatch (50%H) and ii) the osmotic pressure of each solution (O.P.) used. Each value was obtained from 6 replicates of 30-50 eggs each.

Treatments	Concentrations (molar)					
	0.5M	0.25M	0.12M	0.06M	0.03M	H ₂ O
NaCl/Buffer						
O.P. (mOsm/Kg)	947	514	296	192	130	
50%H (Hrs.Mins.)	-	-	-	18.45.	15.30.	12.30.
Sucrose/Buffer						
O.P. (mOsm/Kg)	677	338	201	137	104	
50%H (Hrs.Mins.)	-	-	20.45.	14.30.	12.30	
NaCl/H ₂ O						
O.P. (mOsm/Kg)	825	410	202	90	43	
50%H (Hrs.Mins.)	-	-	21.30.	13.30.	11.30.	
Sucrose/H ₂ O						
O.P. (mOsm/Kg)	595	278	131	65	33	
50%H (Hrs.Mins.)	-	-	15.15.	12.15.	12.30.	
Buffer						
O.P. (mOsm/Kg)	784	390	200	111	57	
50%H (Hrs.Mins.)	-	-	19.30.	13.15.	12.45.	

concentration of 58 and 111.5 mOsm/kg respectively. The time taken for eggs to reach 50% hatch, where measurable, increased with increasing osmotic pressure of the solutions (Table 7). The optimum rate and level of hatching occurred in eggs incubated in solutions of approx. 100mOsm/kg or less.

Acid, Enzymes, Bile and Reducing Agents.

Materials & Methods

The effect of HCl, pepsin-HCl, trypsin, bile, and the reducing agents dithiothreitol, sodium dithionite and cysteine on the hatching of *A.tetraptera* eggs was examined. In each of the concentrations of the various reagents given below, six replicates (approx. 50 eggs each) were incubated for the following periods 0.5hr, 1hr, 2hr and 4hr prior to incubation in 0.05M Bicine buffer (pH 8.5) overnight at 37°C. An equivalent number of replicates was also incubated continuously at 37°C in each of the concentrations of the different reagents. As a control for each treatment, a further batch of replicates was incubated in 0.05 Bicine buffer (pH 8.5) only at 37°C for the length of the experiment. For each period of incubation examined the replicates for the different concentrations of a particular reagent were incubated in Nunc microwell plates 96 (Gibco Ltd.). In order to assess the effect of the reagents on hatching, the mean \pm SE total percentage hatch, the mean time to 50% hatch and whether 50% full emergence was obtained was calculated for each concentration and incubation period examined.

HCl

The following molar concentrations of acid, 0.1M, 0.05M, 0.01M, 0.005M, and 0.001M were prepared from 1N HCl and glass distilled water. The results are presented in Table 8.

Pepsin-HCl

Pepsin concentrations of 10%, 5%, 1%, 0.5% & 0.1% were prepared from Pepsin A powder (porcine stomach mucosa, Sigma Ltd.) in 0.005M HCl (optimum acid concentration for hatching obtained from Table 8). The results are presented in Table 9.

Trypsin

Trypsin concentrations of 5%, 1%, 0.5% & 0.1% were prepared using trypsin Type I, from Bovine Pancreas (Sigma Ltd.) in 0.05M Bicine buffer (pH 8.5). The results are presented in Table 10.

Bile

Bile concentrations of 10%, 5%, 3%, 1% & 0.5% were prepared from Bovine bile (Ox gall powder, Sigma Ltd.) in 0.05M Bicine buffer (pH 8.5) solutions. The results are presented in Table 11.

Reducing Agents

Sodium dithionite, cysteine and dithiothreitol (all obtained from Sigma Ltd.) concentrations of 10%, 1%, 0.1%, 0.01%, 0.001% & 0.0001% were prepared in 0.05M Bicine buffer (pH 8.5). Two batches of replicates were prepared for each of the different concentrations of the three reducing agents in microwell plates. The one batch was incubated as described in the materials and methods under aerobic conditions. The replicates of the other batch were incubated in solutions of the reducing agents previously gassed with 100% CO₂ for 15 minutes and left in a desiccator with an atmosphere also gassed with CO₂ for a further 15 minutes. A layer of paraffin oil was added to each well to prevent diffusion of oxygen into the solutions. The plates were then incubated under the same conditions as the other batch of eggs. Measurements of the pH values, Eh values and the partial pressures of oxygen and carbon dioxide present in each solution were to be made on termination of the experiment using microelectrodes. Unfortunately spillage of the samples in transit prevented this development. The results are presented in Table 12.

Dithiothreitol

As dithiothreitol was found to have a stimulatory effect in the above experiment on hatching, the effect of concentrations of 0.1-0.01% dithiothreitol in 0.05M Bicine buffer (pH 8.5) at 37°C on hatching was observed. The results are presented in Table 13.

Results

HCl [Table 8]

At the higher concentrations of 0.05M and 0.1M HCl, increased incubation suggests a reduction in total hatch. Eggs incubated for 2 hours or longer at these concentrations obtained a lower percentage hatch (less than approx.75%), than eggs incubated in the control of buffer alone ($80 \pm 5.5\%$). Hatching occurred at approximately double the rate of the control, which reached 50% hatch after 10.3 hours. The percentage of larvae which fully emerged in 0.1M and 0.05M HCl was less than about 50% for all periods of incubation. The total hatch of eggs (approx.85 to 95%) incubated in concentrations of 0.001M to 0.01M was higher than the control for all lengths of incubation excepting continuous incubation (approx.45% or less). The time to 50% hatch for each concentration, 0.01M, 0.05M and 0.1M decreased by between 2 and 3 hours with increasing incubation of between 0.5 and 4 hours. Full emergence was less than 50% for eggs incubated continuously at any concentration. Optimum hatching, $92 \pm 5\%$ and $96 \pm 2\%$ occurred when eggs were incubated in either 0.01M or 0.05M HCl respectively for 4 hours at 37°C prior to incubation in 0.05M Bicine buffer (pH 8.5) at 37°C overnight.

Pepsin-HCl [Table 9]

Eggs incubated at concentrations of 0.5 and 1% pepsin/HCl and in 0.005M HCl for between 0.5 and 4 hours obtained an equivalent level of hatch to eggs incubated in the control of buffer alone ($85 \pm 5.5\%$). Reduced levels of hatch, less than about 20%, occurred in eggs incubated continuously in all concentrations of pepsin/HCl and 0.005M HCl only, and in 5% pepsin/HCl for 0.5 to 4 hours ($71 \pm 5.5\%$). The rate of hatch was increased in eggs incubated for all lengths of incubation in 0.5% and 1% pepsin/HCl when compared to 5% pepsin/HCl, 0.005M HCl only and the buffer control. The time to 50% hatch of between 3 and 3.5 hours was achieved after 4 hours incubation in 1% and 0.5% pepsin/HCl. The time to 50% hatch was reached in 6 and 5.5 hours after 4 hours incubation in 5% pepsin/HCl and 0.005M HCl respectively. The control took 13 hours to attain 50% hatch. Less than 50% full emergence occurred in eggs incubated in 5% pepsin/HCl for any period of incubation and at any concentration when incubated continuously. Optimum hatching occurred when eggs were incubated in 1% or 0.5% P/HCl for 4 hours prior to incubation in

TABLE 8

The data presented below gives:- i) the percentage mean \pm SE total hatch (TH%) for eggs incubated in different concentrations of HCl at 37°C for varying lengths of time, prior to incubation in 0.05M Bicine buffer (pH 8.5), at 37°C; ii) the mean time (Hr. Min.) for eggs to reach 50% hatch (50%H); iii) whether less than 50% of the hatched larvae were fully emerged (*) and iv) the optimum concentration/length of incubation (—). Each value was obtained from 6 replicates of 30-50 eggs each.

Length of incubation in HCl solution	HCl concentrations (molar)					
	0.1M	0.05M	0.01M	0.005M	0.001M	H ₂ O
0.5Hr.	TH% 83 ± 4 *	84 ± 2.5 *	89.5 ± 4	91.5 ± 3	85 ± 3	
	50%H 6.0.	6.0.	8.15.	8.15.	9.45.	
1Hr.	TH% 80.5 ± 3 *	75 ± 1.5 *	90.5 ± 2	93 ± 2.5	92 ± 3.5	
	50%H 6.0	5.45.	7.45.	8.0.	10.0.	
2Hr.	TH% 73 ± 5 *	71 ± 2 *	88.5 ± 3	96 ± 3	89 ± 2.5	
	50%H 5.30.	4.45.	6.45.	7.45.	8.30.	
4Hr.	TH% 76 ± 5.5 *	74 ± 4 *	92 ± 5	96 ± 2.5	86 ± 5	
	50%H 5.15.	5.0.	<u>5.30.</u>	<u>5.15.</u>	7.0.	
Continuous	TH% 75 ± 3 *	11 ± 2.5 *	27 ± 4 *	37 ± 3 *	45 ± 4.5 *	80 ± 5.5 *
	—	—	—	—	—	10.30.

TABLE 9

The data presented below gives:- i) the percentage mean+SE total hatch (TH%) for eggs incubated in different concentrations of pepsin in 0.005M HCl at 37°C for varying lengths of time, prior to incubation in 0.05M Bicine buffer (pH 8.5), at 37°C; ii) the mean time (Hr.Min.) for eggs to reach 50% hatch (50%H); iii) whether less than 50% of the hatched larvae fully emerged (*) and iv) the optimum concentration/length of incubation (—). Each value was obtained from 6 replicates of 30-50 eggs each.

Length of incubation in P/HCl solution	Pepsin concentrations (%)				
	5%	1%	0.5%	HCl	buffer
0.5Hr.	TH% 66.5+5.5 *	88+3	83+3.5	82.5+5	
	50%H 13.30	7.45	9.0	10.0	
1Hr.	TH% 59.5+3.5 *	92+3.5	83+3.5	81+5	
	50%H 14.45	8.0	8.15	10.30	
2Hr.	TH% 69+4 *	86.5+3	88.5+3	82+3	
	50%H 12.30	6.15	6.15	9.15	
4Hr.	TH% 71+5.5 *	91.5+2	87.5+3	82.5+4	
	50%H 6.30	3.15	3.0	5.30	
Continuous	TH% 0.5+0.5 *	14+1.5 *	20+4 *	13+1.5 *	85+5.5 *
	50%H -	-	-	-	13.

0.05M Bicine buffer (pH 8.5), both at 37°C, overnight.

Trypsin [Table 10]

Maximum levels of hatch of 83 ± 2 to $87.5 \pm 1\%$, $88 \pm 1\%$ and $94 \pm 1.5\%$ were obtained from eggs incubated for periods of 2 and 4 hours in trypsin concentrations of 1%, 0.5% and 0.1% respectively. Less than 50% of the larvae which hatched were fully emerged from eggs incubated in 5% trypsin for any length of time and 1% and 0.5% trypsin continuously. The time to 50% hatch was increased between 1 and 3 hours in 5% trypsin for all incubation periods when compared with 1%, 0.5% and 0.1% trypsin concentrations. Eggs incubated for 2 and 4 hours showed a decreased time to 50% hatch of 9 and 4 hours respectively when compared to the control (11 hr. 45min.). Concentrations of trypsin of 0.1% to 1% for all periods of incubation decreased their time to 50% hatch, less than 10.5 hours, in comparison with the control, excepting continuous incubation when the time taken to reach 50% hatch was equivalent to the control. Eggs incubated in 5% trypsin continuously had a decreased rate of hatch when compared to the control taking 20 hours to reach 50% hatch. Less than 50% full emergence occurred in eggs incubated in 5% trypsin for 4 hours and when incubated continuously at all concentrations. Optimum hatching was obtained when eggs were incubated in 0.1% or 0.5% trypsin for 4 hours at 37°C prior to incubation in 0.05M Bicine buffer (pH 8.5) overnight, also at 37°C.

Bile [Table 11]

Bile salts increasingly inhibited hatching at concentrations of 3%, 5% and 10% when compared to the control ($93 \pm 2.5\%$). No hatching occurred in 10% bile, irrespective of the length of incubation. In 3% and 5% bile, eggs showed a decrease in mean \pm SE percentage hatch from about 80 to 20% and 70 to 15% respectively with increased incubation from 0.5 to 4 hours. The time to 50% hatch in 3 and 5% bile, where measurable, increased in comparison to the control (14 hrs) by between 1 and 2 hours. No differences were observed in the rate and level of hatch obtained in eggs incubated in bile concentrations of 1%, 0.5% and the control, excepting a reduced level of hatch ($71 \pm 4.5\%$) of eggs incubated in 1% bile solution continuously. Less than 50% full emergence occurred in eggs incubated in 5 and 1% bile after any period of incubation.

TABLE 10

The data presented below gives:- i) the percentage mean \pm SE total hatch (TH%) for eggs incubated in different concentrations of trypsin in 0.05M Bicine buffer (pH 8.5), at 37°C, for varying lengths of time, prior to incubation in the above buffer also at 37°C; ii) the mean time (Hr.Min.) for eggs to reach 50% hatch (50%H); iii) whether less than 50% of the hatched larvae fully emerged (*) and iv) the optimum concentration/length of incubation (—). Each value was obtained from 6 replicates of 30-50 eggs each.

Length of incubation in trypsin solution	Trypsin concentrations (%)				
	5%	1%	0.5%	0.1%	buffer
0.5Hr.	TH%	78.5 \pm 2	82 \pm 2	79.5 \pm 2	80 \pm 1.5
	50%H	12.30.	10.	9.45.	10.
1Hr.	TH%	76.5 \pm 2	82 \pm 1.5	85 \pm 4	77.5 \pm 2.5
	50%H	11.45	10.30.	10.30.	10.30.
2Hr.	TH%	78 \pm 1.5	87.5 \pm 1	88 \pm 1	94 \pm 1.5
	50%H	9.15	7.	7.	7.
4Hr.	TH%	69 \pm 7.5 *	83 \pm 2	88 \pm 1	94 \pm 1.5
	50%H	7.	5.45.	<u>4.15.</u>	<u>4.</u>
Continuous	TH%	68.5 \pm 4.5 *	78.5 \pm 2 *	79 \pm 1.5 *	83 \pm 3 *
	50%H	20.	12.	11.15	10.45
					11.45.

TABLE 11
 The data presented below gives:- i) the percentage mean+SE total hatch (TH%) for eggs incubated in different concentrations of bile in 0.05M Bicine buffer (pH 8.5) for varying lengths of time, at 37°C, prior to incubation in the above buffer, also at 37°C; ii) the mean time (Hr. Min.) taken for eggs to reach 50% hatch (50%H); iii) whether less than 50% of the hatched larvae fully emerged (*) and iv) the optimum concentration/length of incubation (—). Each value was obtained from 6 replicates of 30-50 eggs each.

Length of incubation in bile solutions		Bile concentrations					
		10%	5%	3%	1%	0.5%	buffer
0.5Hr.	TH%	-	72+6.5	80+2.5	90.5+3.5	95.5+3	
		*	*	*			
	50%H	-	14.15	14.15	13.30	14.15	
1Hr.	TH%	-	55.5+2.5	61+4.5	86.5+1.5	88.5+2.5	
		*	*	*			
	50%H	-	16.30	15.30	14.15	13.45	
2Hr.	TH%	-	31+3.5	27+2	89+3.5	89+1.5	
		*	*	*			
	50%H	-	-	-	14.15	14.30	
4Hr.	TH%	-	16+2.5	21+3.5	81+5.5	91+2.5	
		*	*	*			
	50%H	-	-	-	14.30	14.00	
Continuous	TH%	-	1.5+0.5	6.5+2.5	71+4.5	92+3.5	93+2.5
		*	*	*			
	50%H	-	-	-	15.00	13.45	14.00

Table 12

The data presented below gives:- i) the percentage mean+SE total hatch (TH%) for eggs incubated in different concentrations of reducing agents in 0.05M Bicine buffer (pH8.5) in an aerobic environment^(A) and gassed with 100% CO₂^(B) at 37°C, prior to incubation in the above buffer, also at 37°C; ii) the mean time (Hr.Min.) for eggs to reach 50% hatch (50%H) and iii) whether less than 50% of the hatched larvae fully emerged (*) and iv) the optimum concentration/length of incubation (—). Each value was obtained from 6 replicates of 30-50 eggs each.

Length of incubation in reducing agents		Reducing agent concentrations (%)						
		10%	1%	0.1%	0.01%	0.001%	0.0001%	0%
sodium dithionite								
A	TH%	-	-	-	35+2.5 *	68+3 *	86.5+2.5	85+4.5
	50%H	-	-	-	-	14.45.	<u>15.</u>	<u>14.30.</u>
B	TH%	-	-	-	6+4 *	74.5+3.5 *	89.5+2.5	90+3.5
	50%H	-	-	-	-	13.45.	<u>14.30</u>	<u>14.45.</u>
cysteine								
A	TH%	-	6+1.5 *	91.5+2	89+4.5	86.5+3.5	79.5+5.5	85.5+3.5
	50%H	-	-	<u>13.30.</u>	<u>13.30.</u>	13.45.	14.30.	14.45.
B	TH%	-	8.5+3.5 *	92+3	89+3	79.5+2	88+3.5	91+5.5
	50%H	-	-	<u>14.15.</u>	<u>13.45.</u>	13.45.	14.15.	13.30.
dithiothreitol								
A	TH%	-	-	17+7.5 *	95+3	83+3.5	89+8.5	87.5+6.5
	50%H	-	-	-	<u>9.15.</u>	11.30.	<u>13.30.</u>	14.
B	TH%	-	-	12+4.5 *	89+3.5	93+4.5	85+4	85.5+5.5
	50%H	-	-	-	<u>9.30.</u>	12.	14.	14.45.

Reducing agents:- Cysteine, Sodium dithionite and Dithiothreitol [Table 12]

No differences were observed in the effect of any of the reducing agents on the hatching of eggs under aerobic and anaerobic conditions. Cysteine and sodium dithionite failed to stimulate hatching at any concentration, whereas dithiothreitol stimulated hatching at low concentrations. Increasing concentrations of all reducing agents caused decreasing levels of hatch. Sodium dithionite showed increased inhibition when compared to equivalent concentrations of cysteine. Between 10% and 1%, no hatching occurred in sodium dithionite and reduced percentage hatches were obtained in 0.01% and 0.001% of 3.5–6% and 68–74% respectively. The time to 50% hatch (14.5 to 14.75 hrs) and level of hatch (85 to 90%) obtained in a 0.0001% solution of sodium dithionite was equivalent to the buffer control. No hatching occurred in 10% cysteine and reduced hatching of between $6.5 \pm 1.5\%$ and $8.5 \pm 3.5\%$ occurred in a 1% solution. At concentrations of between 0.1% and 0.0001% cysteine, the time to 50% hatch (13.5 to 14 hr) and maximum level of hatch (approx. 85 to 90%) obtained were equivalent to that found in the control. Dithiothreitol inhibited hatching at concentrations of between 10% and 0.1% when compared to the control (approx. 85 to 87%). No hatching occurred in 10% and 1% solutions and reduced hatching of between $17 \pm 7.5\%$ and $12 \pm 4.5\%$ was found in a 0.1% concentration under both anaerobic and aerobic conditions. The rate of egg hatching was stimulated in concentrations of 0.01% and 0.001%, the time taken to reach 50% hatch took approximately 5 and 3 hours less respectively than in the control (14.75 hrs). Less than 50% full emergence occurred from eggs incubated in 0.01 to 0.001% sodium dithionite, 1% cysteine and 0.1% dithiothreitol.

Larvae were found to be active in eggs incubated in concentrations of dithiothreitol of 0.01% or less. No larvae were found to be active in cysteine and sodium dithionite at any concentration when the experiment terminated.

Dithiothreitol [Table 13]

Eggs incubated in 0.1% dithiothreitol showed decreased percentage hatch (approx. 85 to 10%) with increasing length of incubation (0.5hr. to continuous incubation) when compared to the buffer control of $90.5 \pm 2.5\%$. A decreased time to 50% hatch of between 14 and 6.5 hours and between 15 and 11.75

TABLE 13

The data presented below gives:- i) the percentage mean \pm SE total hatch (TH%) for eggs incubated in different concentrations of dithiothreitol in 0.05M Bicine buffer (pH 8.5), at 37°C, for varying lengths of time prior to incubation in the above buffer, also at 37°C; ii) the mean time (Hr.Min.) for eggs to obtain 50% hatch (50%H); iii) whether less than 50% of the hatched larvae fully emerged (*) and iv) the optimum concentration/length of incubation (—). Each value was obtained from 6 replicates of 30-50 eggs each.

Length of incubation in dithiothreitol solution		Dithiothreitol concentration (%)			
		0.1%	0.05%	0.01%	buffer
0.5Hr.	TH%	83.5 \pm 3.5 *	94 \pm 2	89 \pm 1.5	
	50%H	14.30.	14.15.	15.	
1Hr.	TH%	43 \pm 4.5 *	95 \pm 3	92 \pm 3.5	
	50%H	—	10.30.	13.45.	
2Hr.	TH%	22 \pm 6.5 *	91 \pm 4.5	95 \pm 4.5	
	50%H	—	7.15.	12.15.	
4Hr.	TH%	13.5 \pm 3 *	96.5 \pm 2.5	94 \pm 3.5	
	50%H	—	<u>6.45.</u>	11.	
Continuous	TH%	11.5 \pm 4.5 *	92.5 \pm 3	93 \pm 1.5	90.5 \pm 2.5
	50%H	—	<u>6.30.</u>	11.15.	15.15.

hours was observed in eggs incubated in 0.05% and 0.01% dithiothreitol respectively for increasing lengths of time between 1 hour and continuous incubation, when compared to the control (15.25 hrs). The time taken to reach 50% hatch was reduced in 0.05% dithiothreitol when compared to 0.01% dithiothreitol. The maximum levels of hatch achieved in dithiothreitol solutions were equivalent to or higher than the $90 \pm 2.5\%$ hatch obtained in the control of buffer only. The difference between the rate of hatching in the two concentrations increased with length of incubation. Optimum hatching occurred after either four hours or continuous incubation in 0.05% dithiothreitol. Less than 50% full emergence occurred in eggs incubated in 0.1% dithiothreitol continuously. Larvae were only active in solutions of 0.01% or less at the final observation.

Combinations of optimum concentrations of Pepsin-HCl, Trypsin and Dithiothreitol.

The results of the above experiments indicate that incubation of eggs in 1% or 0.5% pepsin-HCl, 0.1% trypsin and 0.05% dithiothreitol for four hours produce an optimum rate and total hatch for each of these reagents. The effect of sequenced combinations of these concentrations on the hatching of eggs incubated for a variety of 4hr and 1hr incubation periods was examined. Bile and trypsin combined were also examined to establish whether these two factors acted synergistically.

Materials & Methods

Three replicates (approx.100 eggs each) were incubated in a 4-well multidish (Gibco Ltd.) at 37°C for each of the treatments investigated as specified in Fig.11. Eggs were incubated in 0.05M Bicine buffer (pH 8.5) overnight at 37°C after incubation in the various series of solutions. Each treatment was examined for evidence of hatching periodically. In order to assess the effect of each treatment on the hatching of eggs, the time to 50% hatch, the proportion of eggs which partially and fully emerged and the total percentage hatch was calculated. The results are presented in Fig.11.

Results

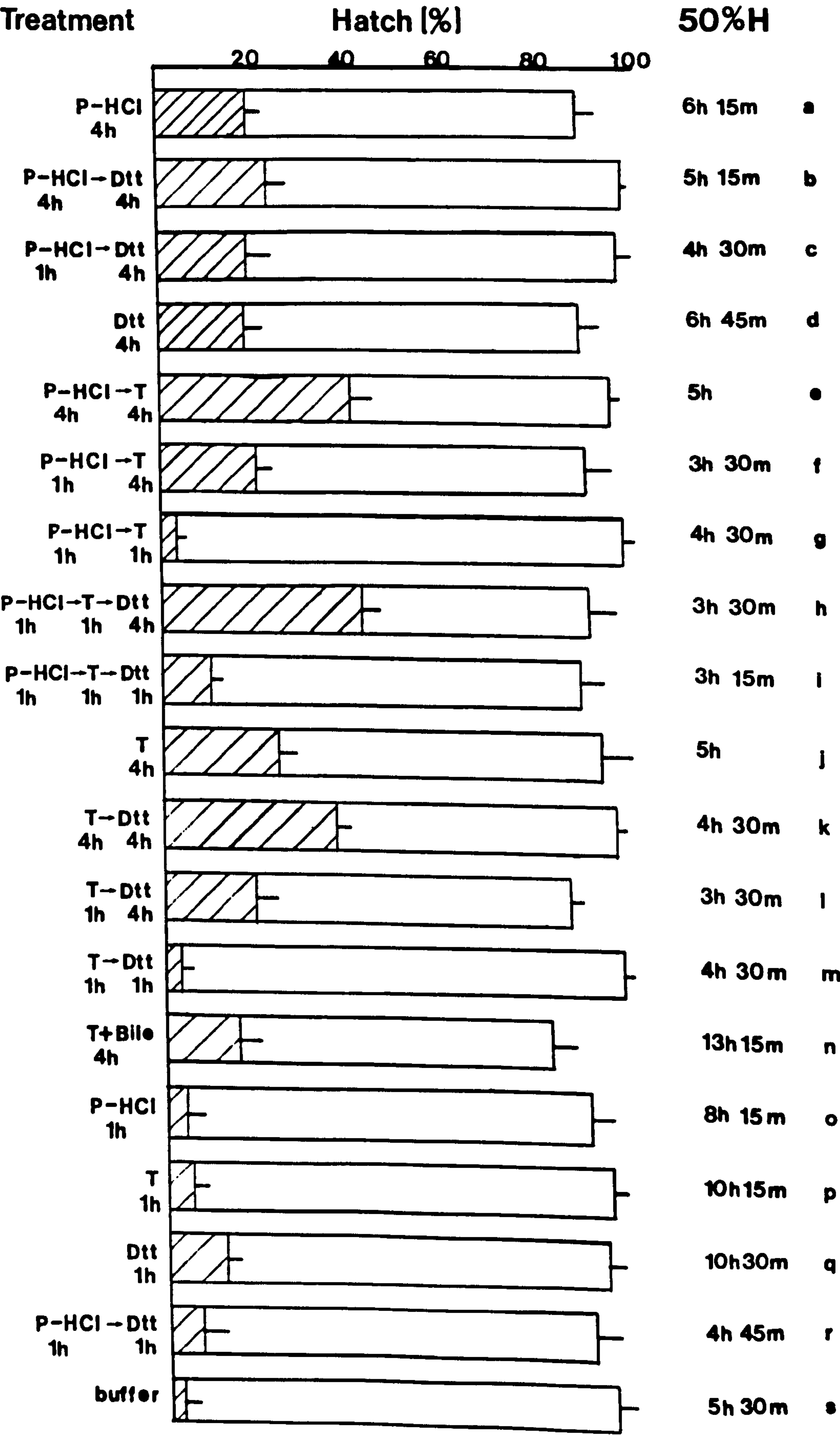
Levels of hatching of over 80% were obtained in all combinations of

pepsin/HCl, trypsin, dithiothreitol and buffer, although the time to 50% hatch and mean \pm SE percentage of larvae which fully emerged varied in different combinations of the solutions. Eggs incubated for periods of 1 and 4 hours in pepsin/HCl, trypsin and dithiothreitol prior to incubation in buffer, showed increased rates of hatch respectively when compared to the control. The time taken to obtain 50% hatch in the control was 13hr 15min compared to between 8hr 15min and 10hr 30min for the three solutions after 1 hour's incubation and between 6hr 45min and 5hr after 4 hours incubation. Eggs incubated in more than one combination of the hatching stimuli for varying incubation periods of 1 and 4 hours further increased their rate of hatch, taking between 3hr and 5hr 15min to reach 50% hatch. In similar combinations of hatching stimuli a 4 hour incubation period in pepsin/HCl and trypsin increased the time to 50% hatch compared to a 1 hour incubation period, Fig. 11a/o and j/p are two examples. An increase in the period of incubation in dithiothreitol in combination with other hatching stimuli did not alter the rate of hatch obtained. Eggs incubated in various sequences of pepsin/HCl, trypsin, dithiothreitol and buffer of 1 hour only in each solution, obtained higher percentages of fully emerged larvae than eggs incubated in similar sequences involving an incubation period of 4 hours in any solution. Examples are presented in Fig. 11a/g, b/r, e/f and h/i.

Fig.11.The effect of pepsin/HCl, trypsin and dithiothreitol on hatching.

i) The mean \pm SE percentage of larvae which partially (☒) or fully (☐) emerged from eggs incubated in 0.05M Bicine buffer (pH 8.5) at 37°C following sequential incubation in a combination of the following factors, pepsin/HCl (P-HCl), trypsin (T) and dithiothreitol (Dtt) for periods of 1(1h) and 4(4h) hours at 37°C.

ii)The time to reach 50%hatch (50%H) in hours (h) and minutes (m). For each value, n=6 replicates of 50 eggs each.



4.2.3 Discussion

The results on hatching of *A.tetraptera* eggs *in vitro* and *in vivo* are discussed in the context of the intestinal environment of the mouse and the results obtained by Anya (1966) *in vitro* and *in vivo*. How these results relate to hatching in other oxyurid and nematode eggs in general is discussed in the final discussion (Section 5). The mechanism of hatching *in vitro* is investigated in the following chapter (Section 4.3.).

Difficulties encountered in obtaining equivalent levels and rates of hatch using optimum hatching conditions devised by Anya (1966) led to a re-examination of the factors he found influenced hatching, that is pH, temperature and osmotic concentration. The hatching data presented by Anya (1966), always combined the number of free and partially emerged larvae in the incubating medium. In the present study, the percentage of free and partially emerged larvae which occurred was presented separately when required in order to assess more accurately the effect of a treatment on hatching.

Anya (1966) investigated the effect of pH on the hatching of eggs within the pH range 1 to 10 at 37°C. Preliminary experiments had indicated that at equivalent pH there was no significant difference in hatching in phosphate, tris, veronal acetate, citrate and borate 0.1M buffers. He found hatching of over 50% occurred in the pH range 6 to 10 after 8 hours incubation at 37°C, with optimum hatching of 95% occurring at pH 7.3 to 7.4. In the present study, the rate and level of hatch achieved in a selection of these buffers at pH range 2 to 10 was less than obtained by Anya (1966). After 8 hours incubation at 37°C, the level of hatch was less than 10% at any pH. After 20 hours incubation maximum hatching reached between 50 and 55% in the pH range 6 to 10, no specific peak was observed between pH 7 and 8. Over 50% of the hatched larvae were only partially emerged. Less than 50% of the hatched larvae were fully emerged.

An increase was observed in the total hatch and the number of larvae which fully emerged when eggs were incubated in a selection of biological 0.05M buffers (pH range 6-10) devised by Good *et al* (1966). The rate of hatch remained relatively unchanged from that which occurred in "Anya's" buffers.

Less than 20% hatch was obtained in any buffer after 8 hours incubation. After 20 hours incubation the total hatch increased to over 75% in the pH range 6 to 9, with maximum hatching of $90 \pm 5\%$ occurring in Bicine buffer, pH 8.5. The number of larvae which fully emerged increased to between 60 and 80% in the pH range 7.5 to 9.5.

The decreased level of hatching of eggs in 0.1M "Anya's" buffers as compared to 0.05M "Good" buffers may be the result of an osmotic effect, described later or a difference in the properties of the buffers. Many of the commonly used buffers such as phosphate, tris and borate are prone to active participation in many biological reactions, complexing a wide range of organic compounds and metabolites (Gueffroy, 1978). The biological buffers were designed by Good *et al* (1966) towards the following criteria:- a pKa between 6 and 8; high solubility in aqueous solutions; exclusion by biological membranes; minimal salt effects; minimal effect on dissociation due to concentration, temperature and ionic composition; well defined or non existent interactions with mineral cations; chemical stability; insignificant light absorption and easily available in the pure form.

The effect of temperature on the level of hatching was similar in both the present investigation and Anya's (1966), with optimum hatching occurring at between 37 and 40°C. However the rate of hatching was reduced, with eggs incubated at temperatures of 37°C or less taking 20 hours to reach equivalent levels of hatch (over 80%) to those obtained by Anya (1966) at similar temperatures after only 8 hours incubation. Although Anya (1966) found maximum hatching occurred between 30°C and 45°C, the high percentage of fully emerged larvae (over 75%) observed at 37°C in comparison with the other temperatures in the present study indicates that this temperature is optimum for hatching. The ability of larvae to hatch at 21°C over a 48 hour period after brief stimulation (1 hour minimal) at 37°C, suggests that the larvae may be involved in the hatching mechanism. This is investigated in Section 4.3.

Anya (1966) found increased inhibition of hatching occurred with increasing molar concentration from 0.1-1M when eggs were incubated in NaCl and sucrose solutions made up in 0.1M veronal acetate buffer (pH 7.3-7.4) and NaCl prepared in buffered Fenwick's saline solution. A similar trend was

observed in the present study when eggs were incubated in NaCl and sucrose solutions of 0.03M to 0.5M concentration, made up in water and Bicine buffer (pH 8.5) and in buffer alone at similar concentrations. The number of larvae which fully emerged also decreased with increasing molar concentration in each treatment.

Anya (1966) reported that at equivalent molar concentrations inhibition was highest in the pure sodium chloride solution. He suggested that this was due to the presence of sodium chloride alone having a toxic effect on living cells (Lockwood, 1960) as opposed to an osmotic effect. Anya (1966), it appears, assumed that the osmotic effect of the different solutions was equal at equivalent molar concentrations. However, measurement of the osmotic pressure of different solutions to assess their effect on hatching showed that solutions of equal molar concentration could differ markedly. For example, hatching in 0.06M NaCl/buffer (192.5mOsm/kg) showed increased inhibition when compared to hatching in 0.06M buffer (111.5mOsm/kg) only. However, the inhibition was far less marked if osmotic pressures were compared, that is 0.06M NaCl/buffer (192.5mOsm/kg) and 0.125M buffer (200mOsm/kg). Therefore the inhibition that Anya (1966) observed in NaCl solutions in comparison to sucrose or Fenwick's solution at equivalent molar concentrations may be the result of osmotic stress as opposed to the toxic effect of the NaCl solution.

In the present investigation optimum hatching occurred in all the solutions at osmotic pressures of about 140mOsm/kg or less. Reduced hatching and decreased full emergence were observed in NaCl/water and sucrose/water when compared to NaCl/buffer and sucrose/buffer at equivalent osmotic pressures. This difference may be attributable to the pH values of the respective solutions, with increased hatching occurring at pH 8.5 in buffer as opposed to pH 5.8 in water. It is unlikely to be due to the toxic effect of pure NaCl solution as a similar trend was apparent in the different sucrose solutions. The rate of hatch increased with increasing osmotic concentration in all the solutions as measured by the time to 50% hatch.

Different results were obtained to those found by Anya (1966) on the action of different concentrations of sodium dithionite and cysteine on the hatching of eggs. He found that the presence of a 1% concentration of sodium dithionite and cysteine had no effect on the hatching of eggs in veronal

acetate buffer (pH 7.3-7.4) under aerobic conditions. In the current investigation increasing concentrations of all the reducing agents were observed to be increasingly inhibitory to hatching. Hatching was completely inhibited in 10% and 1% sodium dithionite and 10% cysteine solutions. As Anya (1966) only investigated the effect of sodium dithionite and cysteine under aerobic conditions, he suggested that the stimulation of hatch by reducing agents might occur under anaerobic or near anaerobic conditions. The results presented in Table 12 indicate that this is unlikely as no differences were observed in the hatching of eggs under anaerobic and aerobic conditions for any of the reducing agents at any concentration. Unfortunately exact details of the microenvironment surrounding the eggs concerning pH and partial pressures of oxygen and carbon dioxide were unavailable due to the circumstances previously described. However due to the extended period of gassing of the solution with 100% CO₂, it has been assumed that near anaerobic conditions had been attained.

The effect of different concentrations of dithiothreitol for varying periods of incubation on the inhibition and stimulation of hatching of eggs is shown in Tables 12 & 13. Inhibition of hatching occurred in eggs incubated in solutions of over 1% concentration. The rate of hatching was stimulated in concentrations of less than 0.01%, although the level of hatch remained equivalent to the control. Optimum hatching occurred when eggs were incubated for 4 hours in 0.05% dithiothreitol, although larval activity was only observed in solutions of 0.01% dithiothreitol or less. The failure of the reducing agents, sodium dithionite and cysteine to stimulate hatching suggests that the effect of dithiothreitol on the eggs and/or larvae may be due directly to the chemical itself, as opposed to its effect on the reduction-oxidation potential of the egg environment.

Any^{ti}a (1966) suggested that temperature and pH were the major factors initiating hatching in *A.tetraptera* eggs. However the slow rates of hatch obtained in the present study, when investigating the effect of temperature and pH on hatching (20 hours for total hatch), indicate that other physico-chemical factors present in the host alimentary tract are involved *in vivo*. Therefore hatching *in vivo* was reinvestigated to clarify the rate and site of hatching. Philpot (1924) obtained free larvae from the posterior end of the small intestine and caecum 4 hours post infection, however no details were

given of dosage levels administered, larval recovery or state of hatching. Anya (1966) found free larvae in the last 5-10 cm of the small intestine and in the caecum of the host two hours post infection. As no larvae were recovered from the stomach or upper intestine, Anya (1966) concluded that these sites were unfavourable for hatching. His findings were based on i) observations obtained from mice infected with low dose rates (100 eggs/mouse) and ii) data pooled from three mice which may have disguised any individual variation present between the mice. In the present study, the number of eggs available prevented replication of results for each observation, however the increased frequency of observations made in the first hour post infection and the high percentage recovery of eggs from the large numbers administered (800 eggs/mouse) allowed a detailed assessment of the site of hatching in the gut.

In comparison to the results obtained by Anya (1966), partially emerged larvae and both partially and fully emerged larvae were recovered from the stomach and the whole length of the small intestine respectively, 15 minutes post inoculum. The majority of freed larvae were found in the caecum between 30 minutes and 1 hour post infection and in the large intestine 2 hours post infection. The number of fully emerged larvae recovered were increasingly reduced in observations made between 2 and 8 hours post infection in comparison to the number of empty eggshells recovered. One explanation for this reduction is the migration of larvae into the crypts of Lieberkuhn of the colon, reported to occur within 24 hours by Behnke (1974) and between 36 and 48 hours by Anya (1966). The number of empty eggshells recovered decreased between 4 and 8 hours post infection and is likely to be due to their poor recovery from the host faecal matter using the salt flotation technique. The increased rate of passage of eggs through the host gut when compared with Anya's results (Anya 1966) may be due to differences in the age and species of mouse used.

The presence of partially emerged larvae in the mouse stomach and fully emerged larvae along the whole length of the small intestine indicated that eggs possibly required exposure to the acid conditions of the stomach prior to the more neutral conditions of the small intestine. Therefore various factors known to be present in the host gut were investigated, including HCl, bile and the enzymes, pepsin and trypsin in an attempt to elucidate further stimuli involved in egg hatching. The effects on hatching of incubating eggs in the

different concentrations of the above factors for varying periods of time were evaluated. Initially the reagents were examined individually to establish the concentrations and periods of incubation required by the eggs for optimum hatching, if appropriate. All the factors examined stimulated hatching at low concentrations except bile. Ox gall powder failed to stimulate hatching at low concentrations and was inhibitory to hatching at higher concentrations. This may be due to the lack of the correct bile acids and salts found in the mouse intestine (Section 4.1). Optimum hatching occurred after 4 hours incubation at 37°C in 0.01/0.005M HCl, 1/0.5% pepsin/HCl, 0.5/0.1% trypsin and 0.05/0.01% dithiothreitol, as indicated by the mean \pm SE percentage total hatch, full larval emergence and the time taken to reach 50% hatch. Increasing concentrations of all these factors inhibited hatching. Sequential incubation in two or more combinations of the above factors at optimum concentrations for 1 and 4 hour periods prior to incubation in buffer increased the rate of hatch. The time to 50% hatch was reached in between 3 and 5 hours depending on the treatment used. The results obtained concerning the hatching of eggs in HCl, pepsin-HCl and trypsin are in agreement with the preliminary studies made by Philpot (1924) and Hsu (1951) who also found emergence was stimulated by these conditions.

Caution must be used in interpreting the results of *in vitro* hatching in terms of hatching *in vivo* in the intestinal environment. However the results obtained in this chapter and by Anya (1966) on the stimulation of hatching *in vitro* are on the whole complementary to the data obtained on the site of hatching *in vivo* and the known physico-chemical and biotic conditions present in the alimentary canal of the mouse (Section 4.2.1.). The optimum pH and temperature for hatching *in vitro* is in agreement with conditions found *in vivo*, that is a temperature of 37°C and a low pH of 3 in the stomach followed by a higher pH of 6-7 in the small and large intestine. The presence of CO₂ and a reducing environment failed to stimulate hatching *in vitro*. However neither have been shown to be stimulatory *in vivo*, (Section 4.2.1). Factors such as HCl, pepsin/HCl and trypsin which stimulated hatching *in vitro* may all be involved in hatching *in vivo*. Hydrochloric acid and the enzymes trypsin and pepsin break up the molecules of protein bonds. Dithiothreitol may affect -SH linkages present in peptide molecules. In order to establish whether these reagents and the reducing agent, dithiothreitol have a direct physical effect on the eggshell or whether the larvae are indirectly stimulated in some manner,

transmission electronmicrographs of eggs stimulated to hatch by these factors are examined in Section 4.3...

To summarize, optimum levels and rates of hatch *in vitro* were obtained in the present study when eggs were incubated sequentially in a variety of combinations of 0.5 to 1% pepsin/HCl, 0.1 to 0.5% trypsin/buffer (pH 6-9) and 0.01 to 0.05% dithiothreitol/buffer(pH 6-9) at 37°C. The time to 50% hatch being reached in 3 to 5 hours and a maximum level of hatch of over 85% being obtained in under 12 hours. Hatching occurred within 1 hour *in viva*. Partially emerged larvae were found in the stomach 15 minutes post infection and the majority of freed larvae were observed in the caecum between 30 minutes and 1 hour after infection. In comparison Anya (1966) obtained optimum hatching *in vitro* (over 80%), when eggs were incubated for 7 hours in "Anya's" buffers only, pH 6-9 at 37°C. He found freed larvae in the posterior region of the small intestine and in the caecum 2 hours post infection when he investigated hatching *in viva*.

4.3 Physiology of hatching

4.3.1 Introduction

This chapter examines the changes which occur in the egg during hatching, using a variety of techniques and attempts to explain the mechanism of hatching. The majority of authors, who investigated hatching in *A.tetraptera* eggs have only examined factors which stimulated hatching (Philpot, 1924; Deschiens, 1944 and Hsu, 1951), as opposed to the hatching process itself. Anya (1966) briefly investigated the possibility that hatching was associated with an increase in the permeability of eggs to water. He found the percentage of plasmolysed eggs increased in a concentrated solution of NaCl after incubation for regular intervals in his artificial hatching medium. Wharton (1979a) investigated the structure and chemistry of the eggshell of the unembryonated egg, as described in Section 3.1., but no observations were made on the eggshell during egg development and hatching.

It was observed in Section 3.2. that i) increased spontaneous hatch occurred with egg age at all developmental temperatures examined and ii) increased osmotic concentration caused larval shrinkage within the egg and affected the ability of apparently "normal" larvae to hatch. These two observations suggested some change occurred in the permeability of the egg prior to hatching. In an attempt to establish if and when a permeability change occurred with age, eggs were examined for larval shrinkage and their ability to hatch after incubation in increasing concentrations of NaCl. From electron microscope studies made in Section 3.3., it was observed that differential staining of eggs occurred in osmium tetroxide. It is a slow penetrating fixative that reacts with components of tissues, particularly lipids, forming osmium black when reduced by unsaturated lipid components of the tissue. The chemistry of the interactions of osmium tetroxide with lipids, proteins, nucleic acids and carbohydrates are summarized in reviews by Millonig & Marinozzi (1968), Riemersma (1970) and Hayat (1975). In this chapter we are concerned with it's function as a stain (Millonig and Marinozzi, 1968). Therefore the pattern of staining in eggs of various ages were used to examine changes in permeability of the eggs prior to hatching at different temperatures. The staining of eggs with Sudan B Black was also undertaken to establish for

certain whether the osmium tetroxide was reacting with lipid complexes within the egg. Sections of differently aged eggs were examined by the transmission electron microscope in an attempt to identify alterations in the eggshell which could be related to the permeability changes previously observed in equivalently aged eggs in concentrated NaCl solutions and osmium tetroxide. An experiment was undertaken in order to examine whether these permeability changes within older eggs increased the rate of hatch once the eggs were stimulated.

In eggs which had not spontaneously hatched, various stimuli were observed to trigger hatching, as described in Section 4.2. Various methods were used to examine the effect of these factors on the eggshell or larvae. Comparative changes in the permeability of the eggs stimulated to hatch by various factors were made by measuring the rate of larval shrinkage in increasing NaCl concentrations using time lapse video recordings and the differential staining of eggs in osmium tetroxide. Eggs at various stages of hatching after incubation in different stimuli were sectioned and examined using the transmission electron microscope. In order to assess changes in the exterior of the eggshell during hatching, eggs were observed at intervals using the scanning electron microscope.

General observations of the hatching behaviour of larvae in the various stimuli and larval activity prior to and during hatching were analysed using the video recording system. Following an experiment which established the length of incubation required by larvae at 37°C for hatching to occur (Section 4.2.3.) an attempt was made to assay hatching eggs for non-specific proteolytic activity using Azocoll dye (Calbiochem-Behring Corp.). Azocoll is an insoluble powdered cowhide, to which a bright red dye is attached. When a proteolytic enzyme breaks any of the peptide linkages of the proteins present in the cowhide, the bound dye is released into the suspending medium. The rate at which the dye is released can be measured spectrophotometrically to assess the enzyme activity in solution.

4.3.2 Experimental procedure and results

For the following experiments, unembryonated faecal eggs were collected and extracted as described in Section 2.1.2. and incubated as detailed in Section 2.1.3, unless mentioned. Hatching was estimated as explained in Section 2.1.4.

The effect of sodium chloride concentrations on larval development, shrinking and hatching in ageing eggs.

Materials and methods

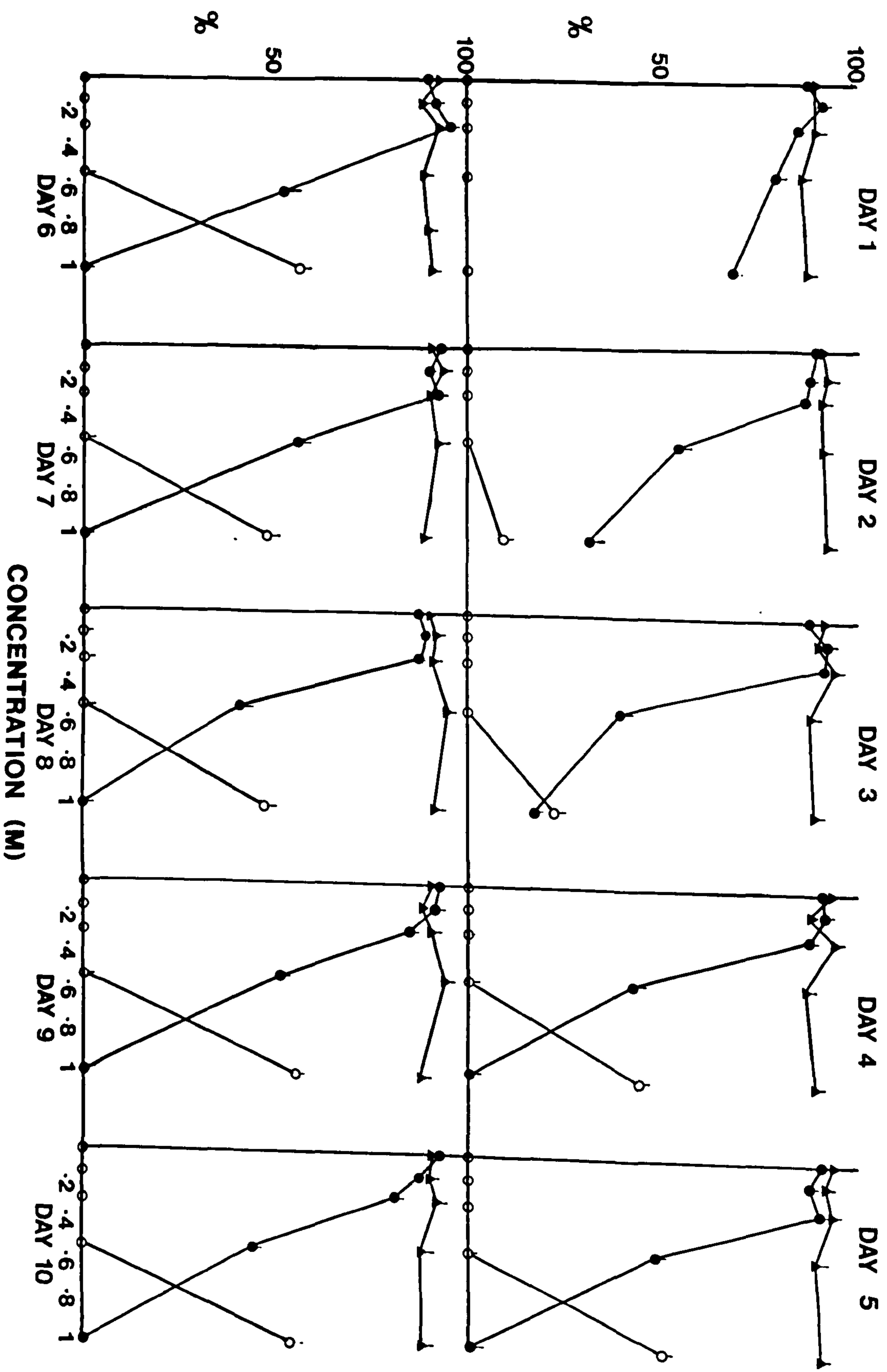
Batches of unembryonated faecal eggs were incubated in the following NaCl concentrations:- 0.12, 0.25, 0.5 and 1M, plus a control of water at 21°C. For each treatment, 10 replicates of 30 to 50 eggs each were examined every 24 hours for evidence of larval development, spontaneous emergence and larval shrinkage. These three characteristics have been described previously in Section 3.2.2. The hatching of eggs *in vitro* was assessed by incubating eggs in 0.05M Bicine buffer (pH 8.5) at 37°C, overnight. The experiment was conducted for a period of 10 days. The results are presented in Fig.12.

Results

The development, shrinking and hatching of differently aged eggs in increasing NaCl concentrations is illustrated in Fig.12. Development was equivalent in all NaCl concentrations and water for the duration of the experiment, between about 87% and 95%. The level of hatching obtained in eggs incubated in increasing NaCl concentrations decreased with time. Optimum hatching of between about 87% and 93% occurred in eggs which had developed in water and 0.12M NaCl throughout the experimental period. After 8 days incubation in 0.25M NaCl, hatching decreased slightly, dropping to $81 \pm 2.5\%$ by day 10. Decreasing levels of hatch were observed in eggs incubated in 0.5 and 1M NaCl, with time. Levels of hatch for eggs incubated in 0.5M NaCl decreased from $80 \pm 3\%$ to $54 \pm 5\%$ between day 1 and 2, and remained between 45 ± 4 and $57 \pm 4.5\%$ from day 3 to day 10. Hatching occurred in eggs incubated in 1M NaCl from days 1 to 3 only, decreasing progressively with time from 68 ± 4.5 to $18 \pm 3\%$. Larval shrinkage was apparent in eggs incubated in 1M NaCl. It increased from $9 \pm 3.5\%$ on day 2, to between 44 ± 2 and

Fig.12.The effect of NaCl concentrations on larval development, shrinking and hatching in ageing eggs.

The mean \pm SE percentage i) development ($\blacktriangle\text{---}\blacktriangle$) and ii) shrinking ($\bigcirc\text{---}\bigcirc$) of eggs incubated in different concentrations of NaCl(0-1M) at daily intervals, plus iii) hatch ($\bullet\text{---}\bullet$) after subsequent incubation in 0.05M Bicine buffer (pH 8.5) at 37°C. For ech value, n=10 replicates of 30- 50 eggs each.



57_±3% from day 4 onwards.

The effect of different lengths of staining in osmium tetroxide on eggs of various ages.

Materials and method

Unembryonated faecal eggs were incubated at 21°C. Seven batches of 10 replicate samples were prepared daily and examined for evidence of larval development and spontaneous emergence (Section 3.2.2.). For each of the following intervals of time:- 0.5, 1, 2, 4, 8 and 12 hours one batch of eggs was incubated in 0.5% osmium tetroxide at 4°C. One batch of eggs was also incubated in water for 12 hours as a control. When examined for staining, two types were observed, egg fluid only and larval only, as depicted in Fig.13a & c. The percentage staining for each was calculated. The samples of eggs were then washed twice in water and incubated overnight in 0.05M Bicine buffer (pH 8.5) at 37°C and hatching was assessed (Section 1.4). A sample of unstained larvae from egg fluid only stained eggs, were pressed from the eggs and incubated in osmium tetroxide at 4°C for 0.5 hour. General observations were made on the activity of unstained larvae and stained larvae within the eggs. The experiment was conducted for a period of 10 days. The results are presented in Fig.14.

Results

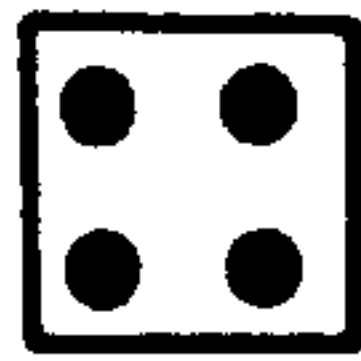
Fig.14. presents the differential staining of eggs aged between 0 and 10 days in 0.5% osmium tetroxide for varying periods of incubation. Increased staining of egg fluid occurred in eggs of all ages incubated in 0.5% osmium tetroxide for all periods of incubation. For eggs of the same age, staining progressively increased with length of incubation in 0.5% osmium tetroxide. Egg fluid staining was found in eggs incubated for 1, 2, 4, 8 and 12 hours in osmium tetroxide after 6.5, 4.5, 3.5, 1.5 and 1.5 days respectively and reached levels of 42_±4.5, 62_±4, 89_±2.5, 87_±3.5 and 92_±1.5% accordingly. Larval staining was found between 7.5 and 10.5 days and reached between about 7% and 12% for periods of incubation between 2 & 12 hours. Hatching occurred in the controls when eggs were aged 1.5 days and over, increasing from 5% to over 80% by day 4.5 and remaining at this level for the rest of the experiment. Stained larvae were observed to be inactive, whereas unstained larvae in egg

Fig.13.Differential types of eggstaining in OsO₄.

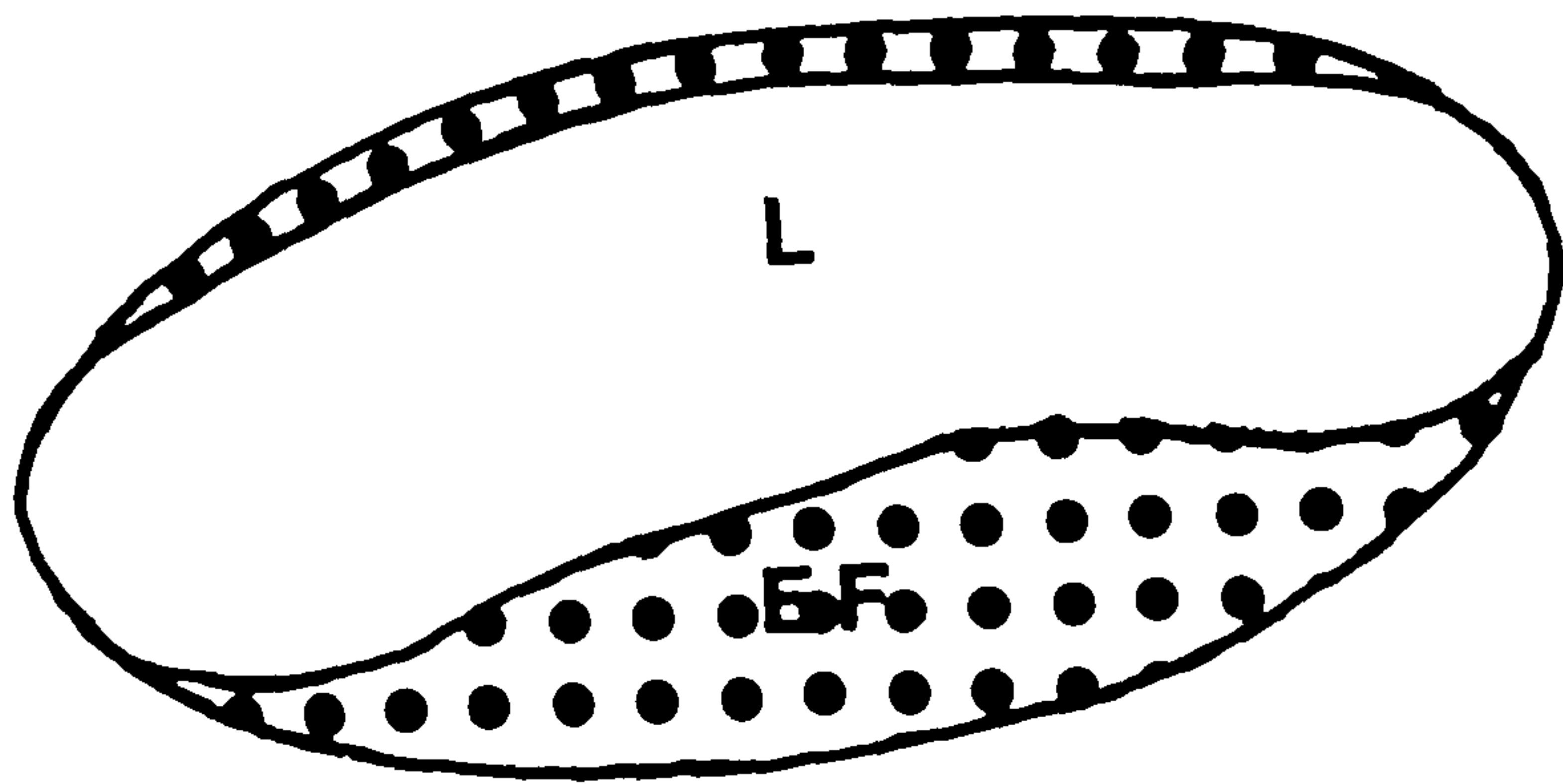
a) Egg fluid only.

b) Egg fluid and larval.

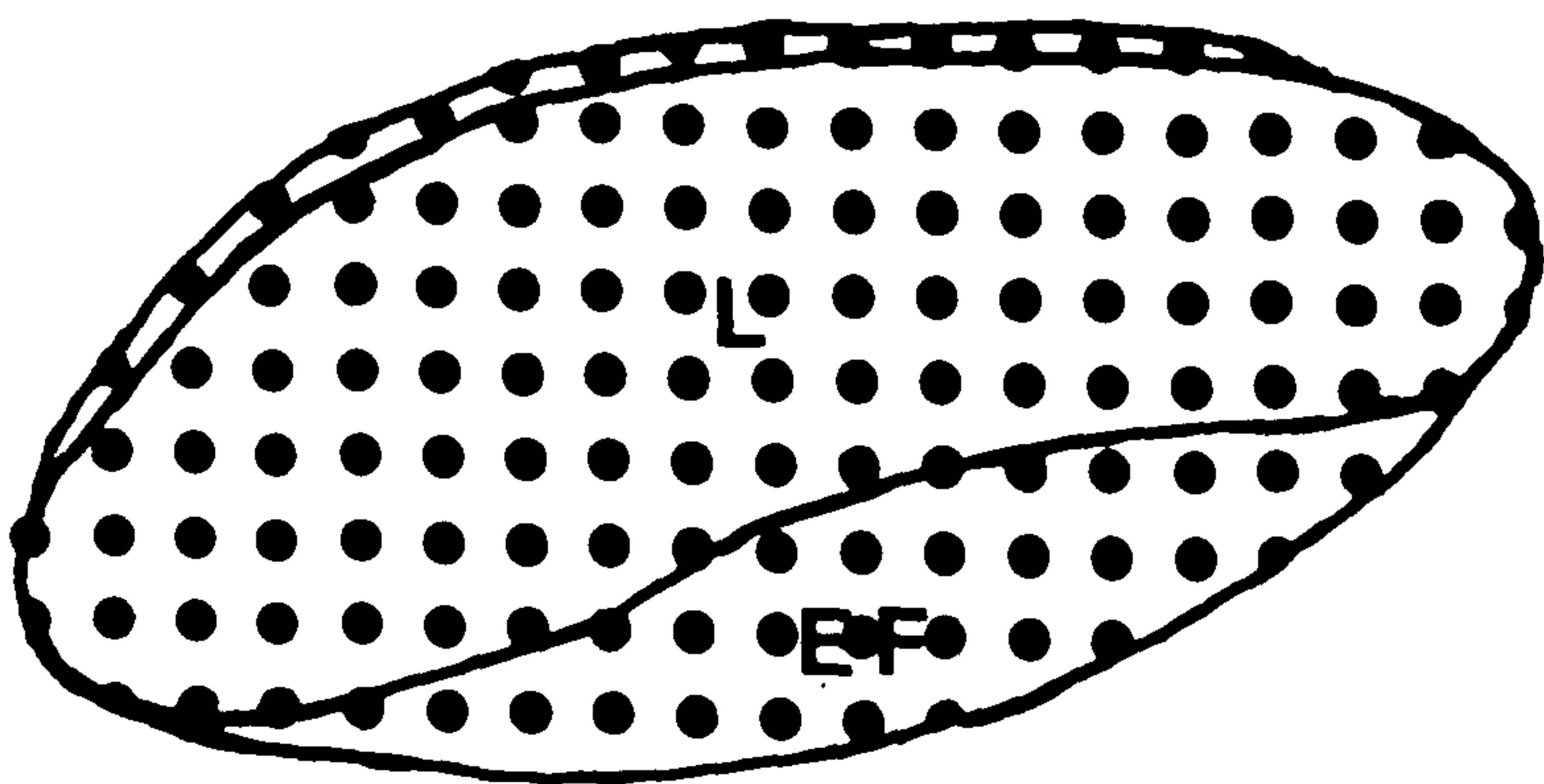
c) Larval only.



A



B



C

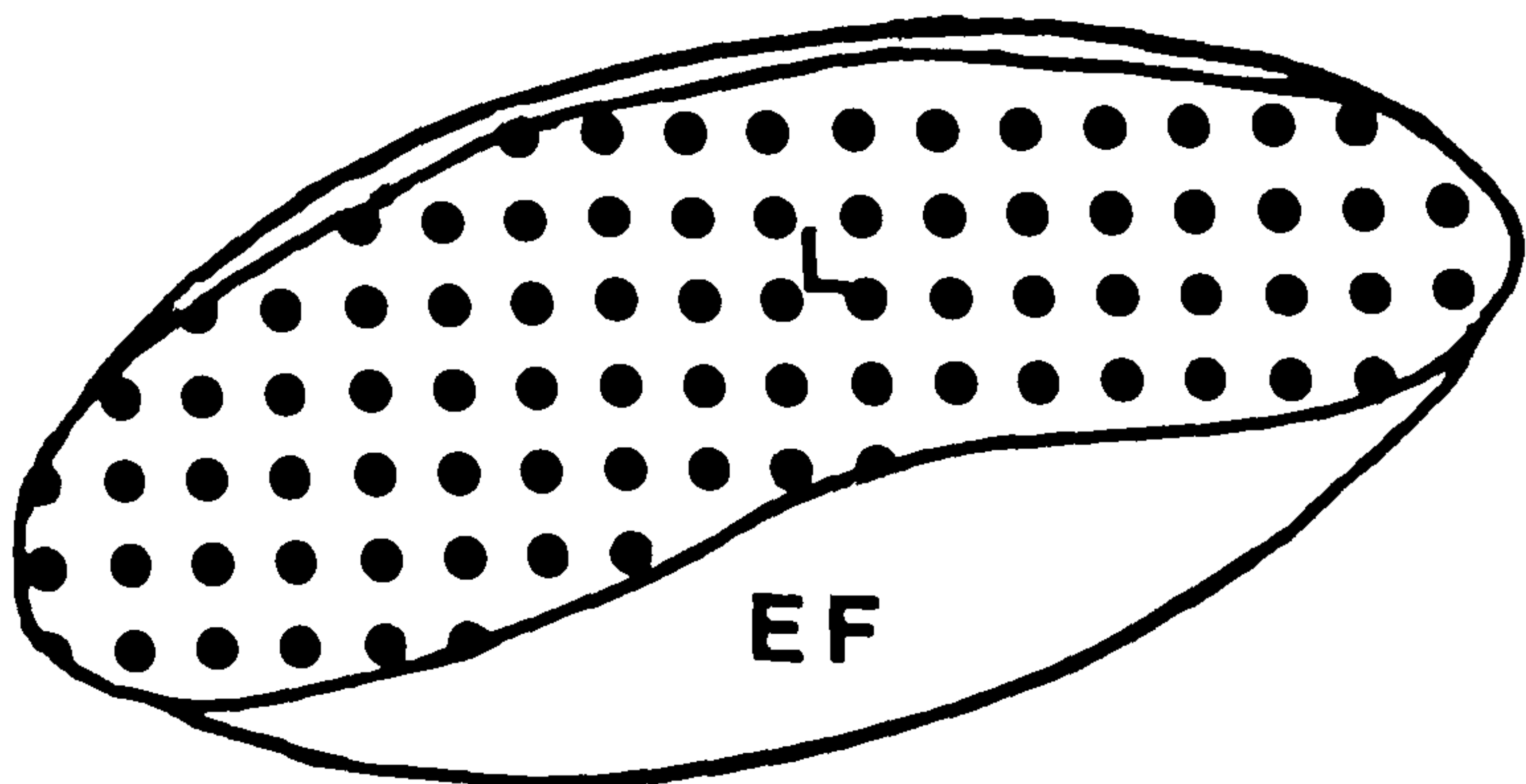
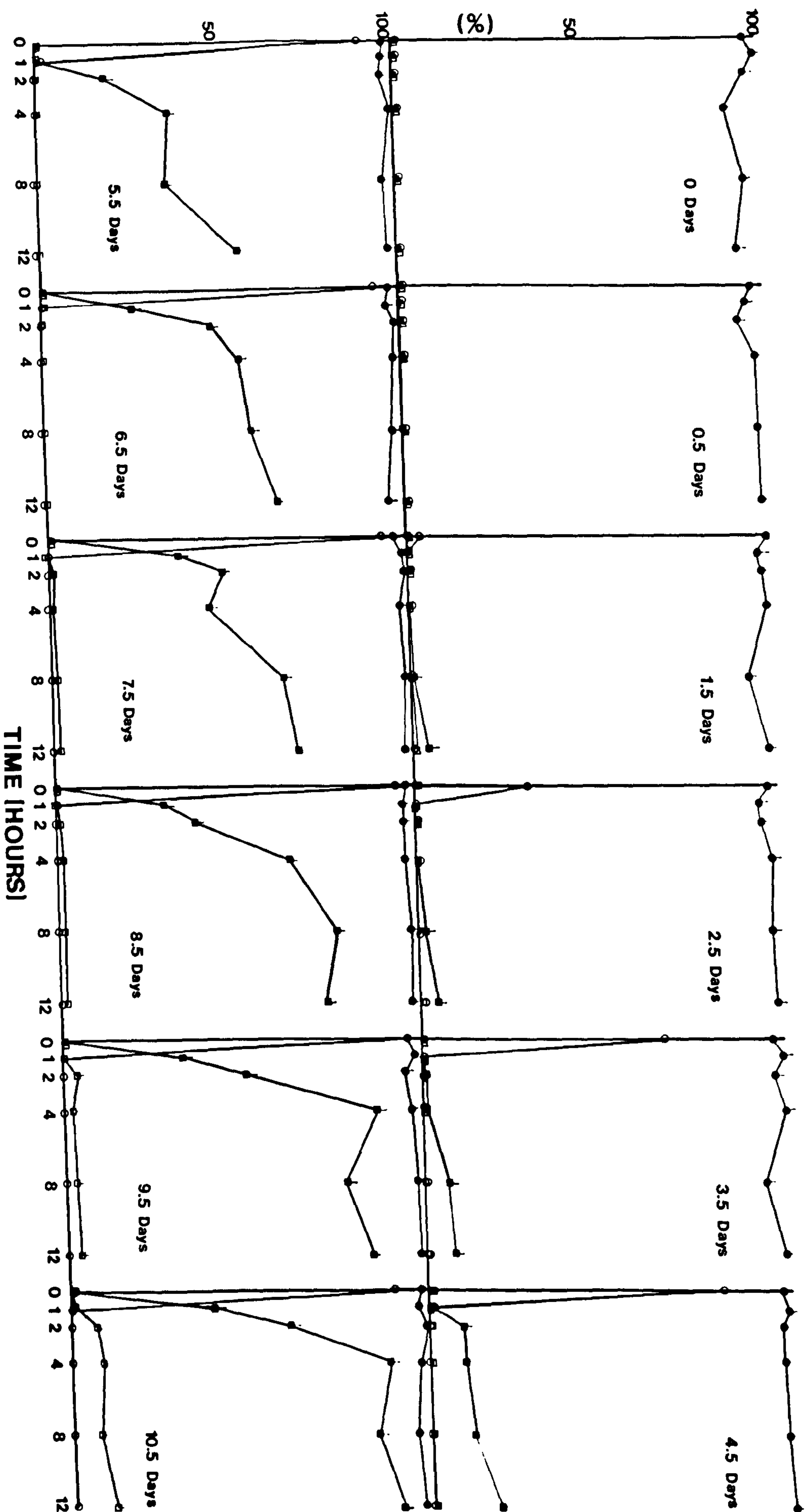


Fig.14.The effect of different lengths of staining in OsO_4 on ageing eggs.

i) Mean \pm SE percentage of eggs with egg fluid only (■—■) and larval only (□—□) staining after incubation for varying lengths of time (0-12 hours) in OsO_4 and ii) mean \pm SE percentage development (●—●) and iii) mean \pm SE hatch (○—○) of eggs incubated overnight in 0.05M Bicine buffer (pH 8.5) at 37°C, after exposure to OsO_4 . For each value, n=10 replicates of 50 eggs each.



fluid only stained eggs were found to be actively wriggling within the shell but no hatching occurred, even after incubation for hatching overnight at 37°C. The operculum remained unstained.

The effect of egg development temperature on the differential staining of eggs of varying ages in osmium tetroxide.

Materials and methods

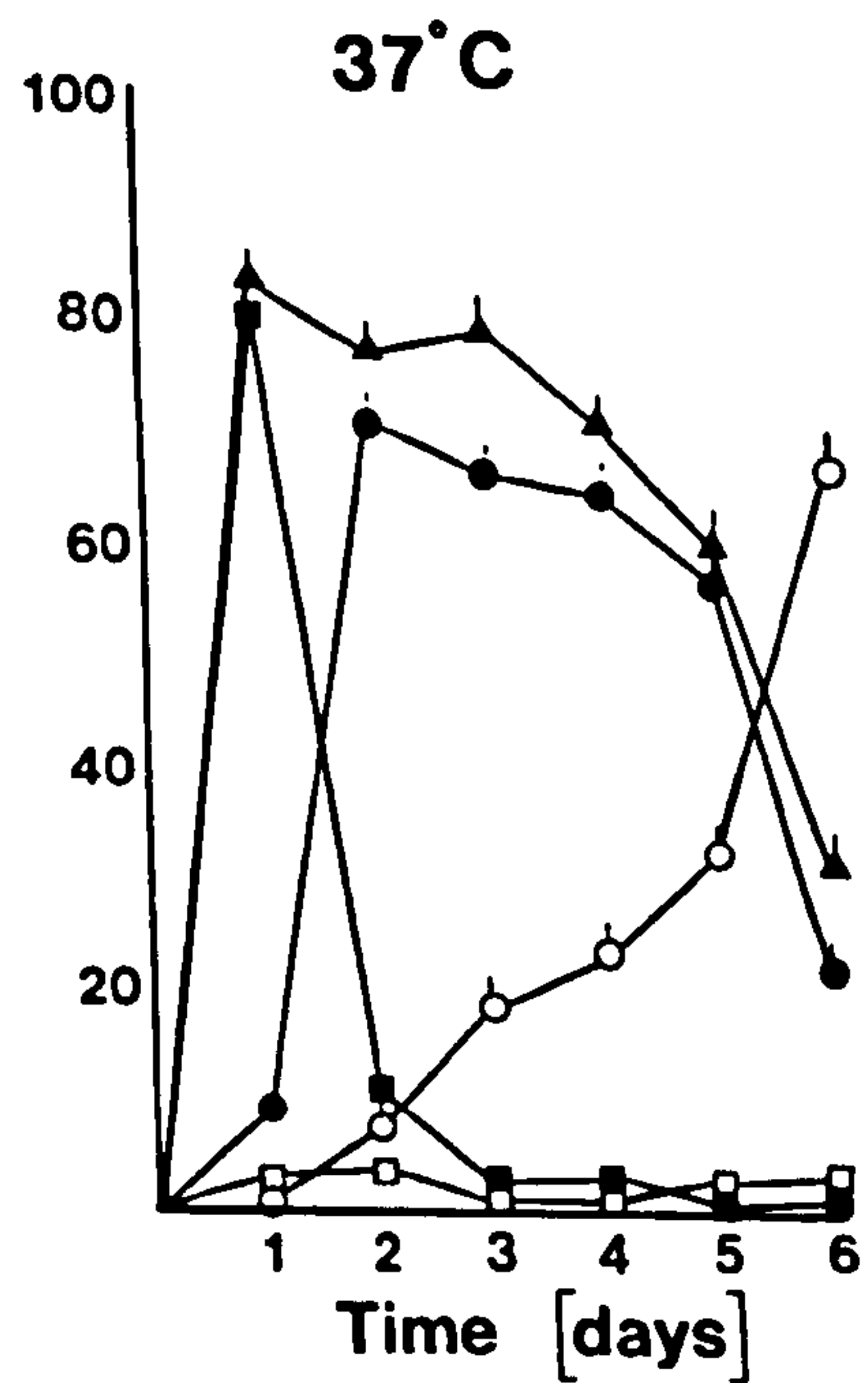
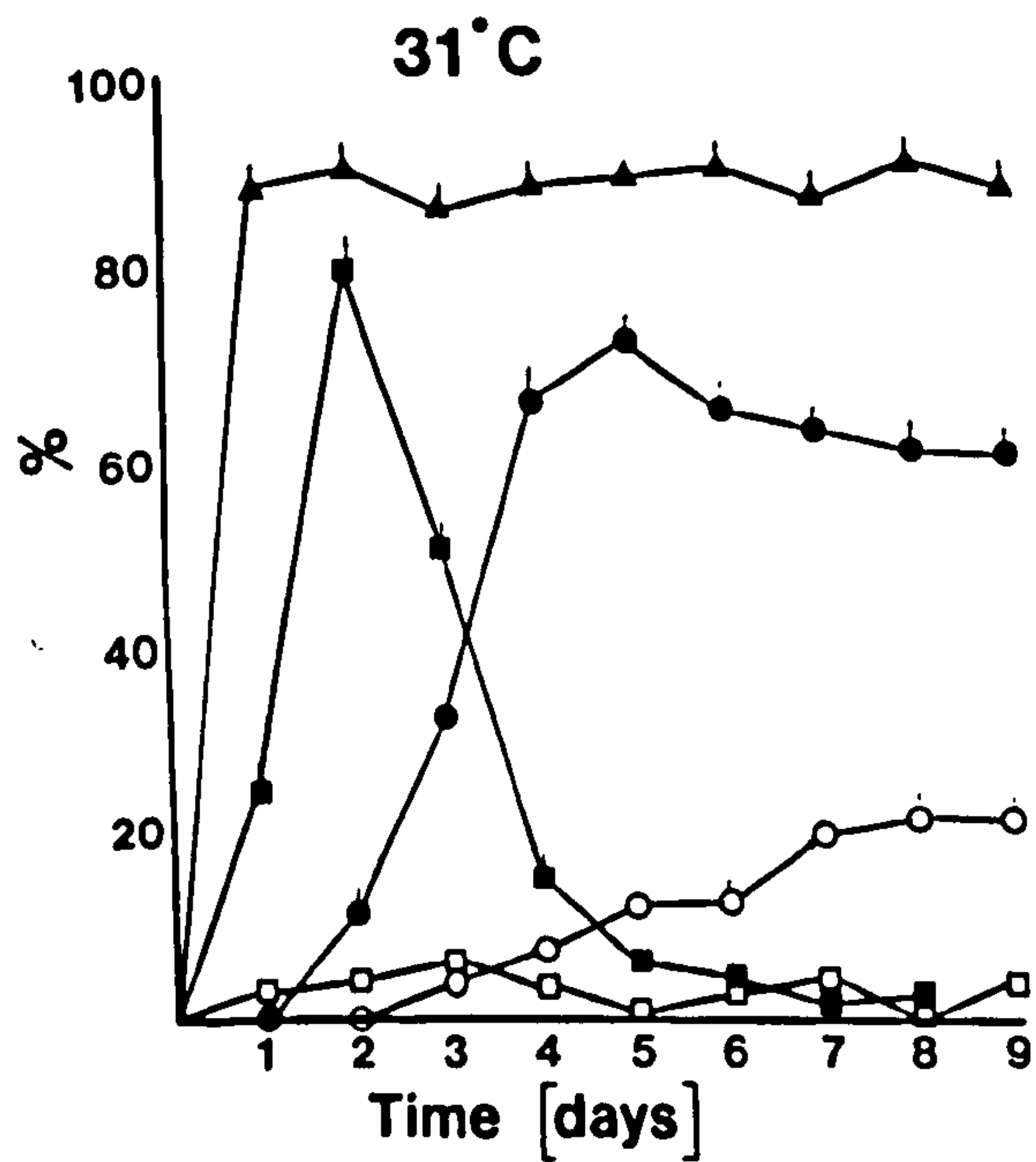
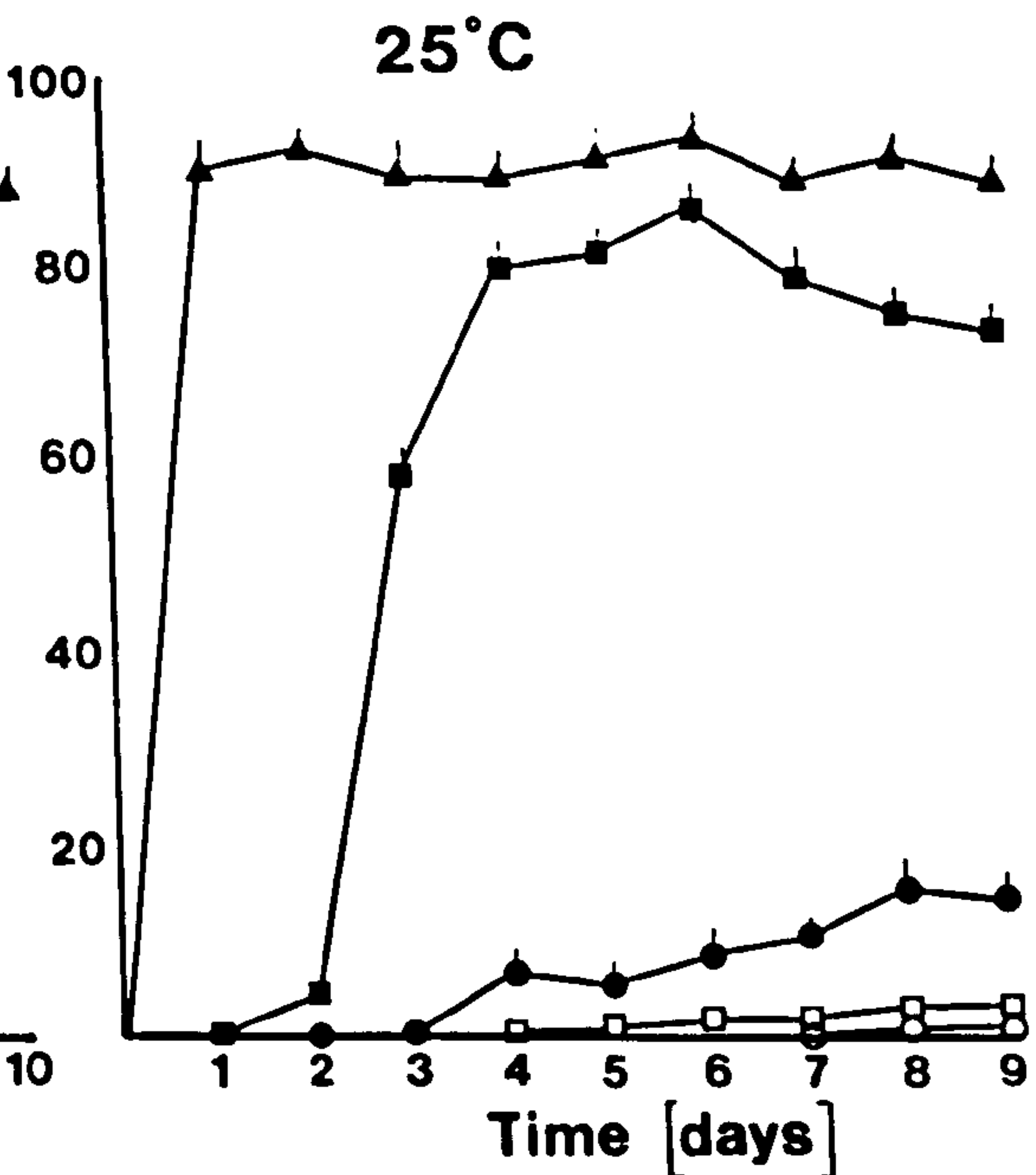
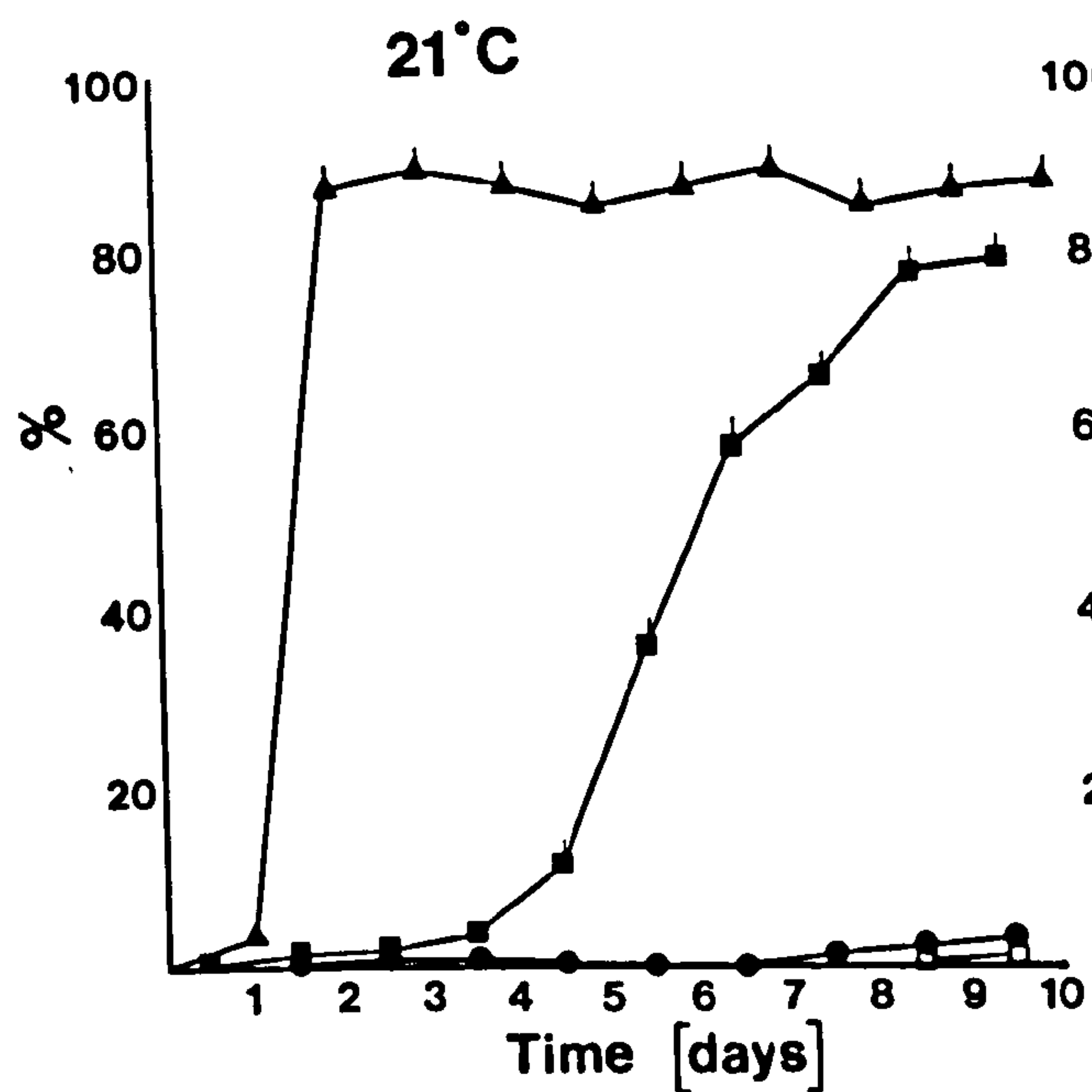
Unembryonated faecal eggs were divided into 4 batches. Each batch was incubated at one of the following temperatures:- 21°, 25°, 31° and 37°C. Every 24 hours, 10 replicate samples of 30 to 50 eggs each were examined for evidence of development and spontaneous emergence (Section 3.2.2.). The eggs were then incubated in 0.5% osmium tetroxide for 12 hours at 4°C. The four types of staining found included i) egg fluid only (Fig.13a); ii) egg fluid and larva combined (Fig.13b); iii) larva within egg only (Fig.13c) and iv) hatched larva. The results are presented in Fig.15.

Results

The differential staining in osmium tetroxide of eggs developed at varying temperatures over a period of 10 days is illustrated in Fig.15. As previously demonstrated in Section 3.3.2., the rate of egg development increased with increasing temperature and spontaneous emergence occurred after a shorter incubation period at increased temperature. Maximum levels of development of between about 80% and 90% remained constant at 21°C, 25°C and 31°C, but 4 to 7 days incubation at 37°C resulted in a decrease in viable larvae. No spontaneous emergence occurred at 21°C, less than 2±1% at 25°C after Day 7. At 31°C and 37°C, levels of emergence reached 21±2% and 60±4% between 2 and 9 days and, 1 and 9 days respectively. Increased temperature resulted in increased levels of staining of eggs of equivalent ages. Levels of staining for egg fluid only had reached between about 75% and 85% after Day 1, 2, 4 and 10 at 37°C, 31°C, 25°C and 21°C respectively. Egg fluid only staining decreased rapidly at 37°C and 31°C reaching levels of between 5% and 10% after Day 3 and 1 respectively. The level of larval staining within the egg correspondingly increased relative to the decrease in egg fluid only staining at each temperature. Maximum levels of larval staining within the egg of about 65% to 70% were found after 2 and 4 days incubation at 37° and 31°C

Fig.15.The effect of development temperature on the staining of ageing eggs in OsO₄.

Mean±SE percentage development (▲—▲) of eggs removed daily from batches incubated at 21°, 25°, 31° and 37°C. Different types of staining observed included eggfluid only (■—■), larval only (●—●), both egg fluid and larval within the egg (□—□) and free larvae (○—○). For each value, n=10 replicates of 30-50 eggs each.



respectively. At 21°C, larval staining in the eggs was low reaching $3 \pm 1\%$ between Day 7 and 10. At 25°C, larval staining increased to 15% between day 3 and 9. A decrease in larval staining occurred within the eggs at 31°C and 37°C, by Day 6 and 9, levels of $23 \pm 4.5\%$ and $60 \pm 2\%$ were obtained respectively. A corresponding increase in free stained larvae was found at 37°C and 31°C, levels of $66 \pm 4\%$ and $21 \pm 2\%$ were reached on Day 6 and 9 respectively. Staining of both egg fluid and the larva within the egg remained at a constant at 31°C and 37°C of less than $5 \pm 2\%$ between Day 1 and 8. Low levels of $2 \pm 0.5\%$ or less were found at 21°C and 25°C after Day 9 and between Day 4 and 9 respectively. Emerged larvae at all temperatures were stained. The contents of non viable eggs stained black, the pattern of staining which occurred in ageing embryonated eggs was not observed.

Staining for lipids in Sudan B Black stain.

Materials and methods

Unembryonated faecal eggs were incubated at 21°C. Every 24 hours, a sample of approximately 100 eggs was removed from the batch and incubated in a saturated solution of Sudan B Black in 70% alcohol for 12 hours at 21°C. The experiment was conducted for a period of 10 days.

Results

No staining^{occurred} in eggs aged 8 days or less. In eggs, aged 9 and 10 days, fewer than 5% of the larvae within the eggs stained a light brown colour. No staining of the egg fluid occurred.

Changes in the eggshell with increasing age

Materials and methods

Unembryonated faecal eggs were incubated at 21°C. Unembryonated eggs and developing eggs (approx. 500 eggs per batch) were removed every 24 hours from the incubation medium and prepared for transmission electron microscopy as described in Section 2.1.5.

Results

Problems were encountered in obtaining whole sections of unembryonated

and immature eggs due to the impermeability of the eggshell to fixatives, therefore only sections of the wall are displayed in Plate 5a and 5b. The layers of the eggshell and the operculum are intact in the unembryonated egg. The eggshell of an egg aged 5 days old is shown in Plate 6a. The internal uterine layer has disintegrated and the chitin and underlying lipid layer have separated from the vitelline layer leaving a gap. The operculum has remained intact (Plate 6b). In Plate 7a, the vitelline layer has also separated from the disrupted internal uterine layer. An egg aged 7 days old is shown in Plate 7b and 8a. The operculum has begun to separate and osmium black deposits can be observed in close proximity to the inner edge of the lipid layer. The chitin layer has separated from the vitelline layer. In an egg, 10 days old, the larva is stained black (Plate 8b). The operculum has begun to disintegrate (Plate 9a), the chitin layer has separated completely from the vitelline layer and the lipid layer has collapsed against the larva (Plate 9b).

The effect of egg age on rate of hatch



Materials and methods

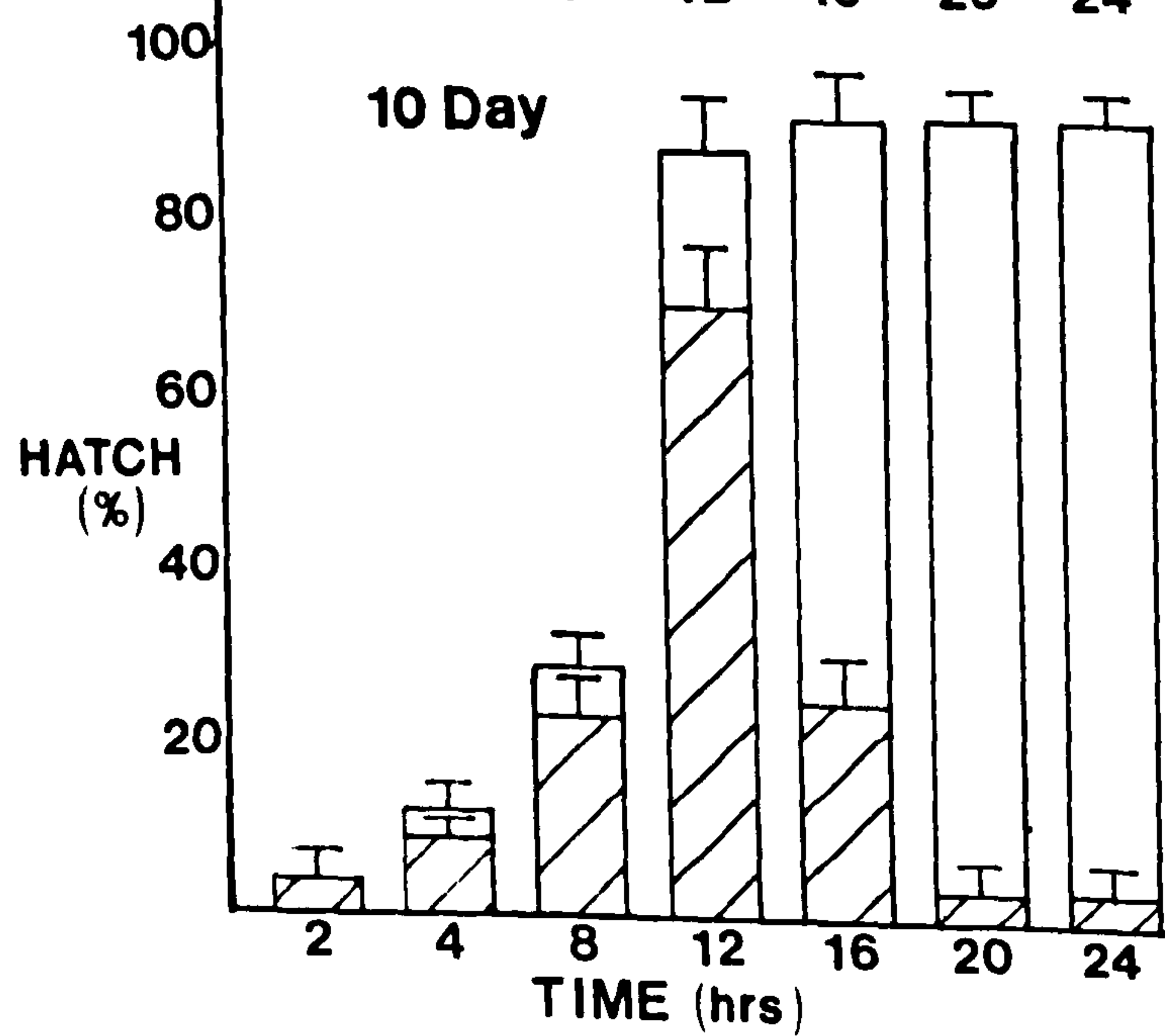
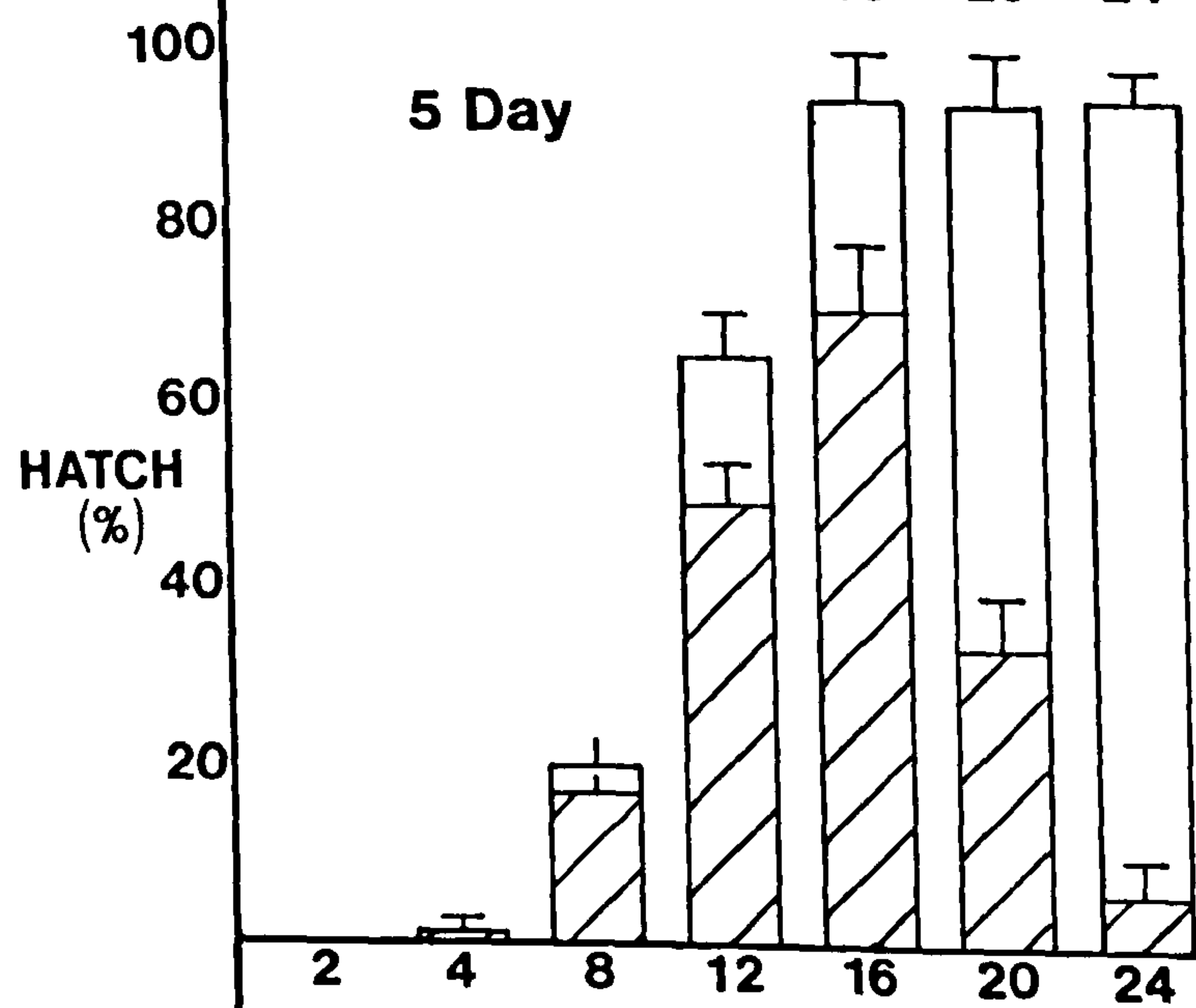
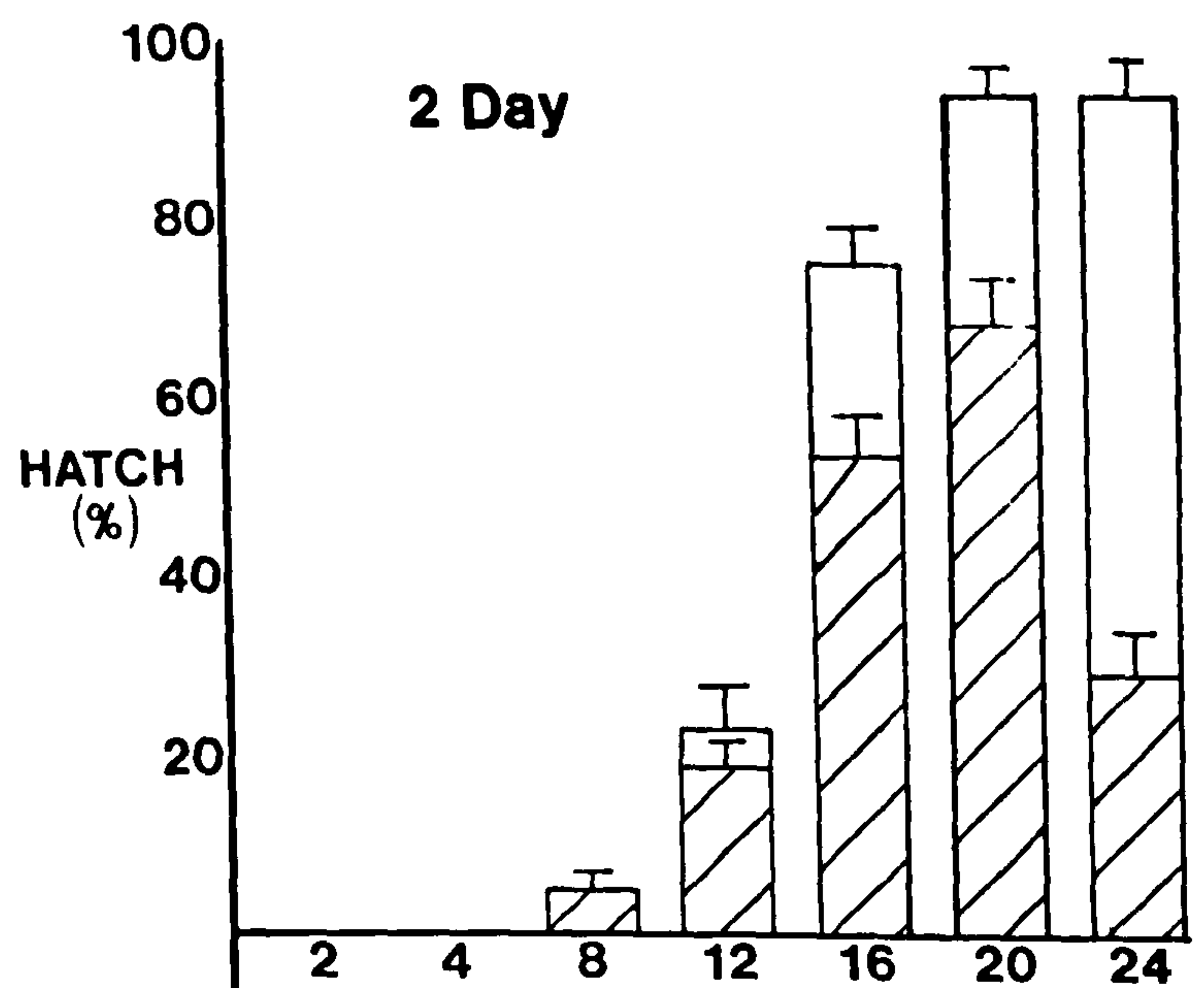
Eggs were incubated at 21°C for 10, 5 and 2 days prior to experimentation. Five replicates of 100 eggs each were incubated in 0.05M Bicine buffer (pH 8.5) at 37°C and examined for evidence of hatching at regular intervals over 24 hours. Samples of 100 eggs each were examined for evidence of background hatching from the differently aged cultures. The results are presented in Fig.16.

Results

The period of time necessary to obtain maximum hatch decreased with the increasing age of the egg populations examined (Fig.16.). Eggs aged 2, 5 and 10 days reached maximum levels of hatch after 20, 16 and 12 hours respectively. All ages of eggs reached an equivalent level of hatch of between about 90 and 95%. An increase in egg age resulted in a reduced period of incubation prior to the onset of larval emergence from the egg. Hatching was initiated in eggs aged 10, 5 and 2 days after 2, 4 and 8 hours respectively. The final number of larvae which fully emerged occurred after 24 hours in 2 and 5 day old eggs and 20 hours in 10 day old eggs. The mean \pm SE percentage full emergence after 24 hours was 65 \pm 5.5%, 88 \pm 4.5% and 91 \pm 3.5% in 2, 5 and 10 day old eggs respectively. No background hatching was observed in 2 and

Fig.16. The effect of egg age on rate of hatch.

The mean \pm SE percentage of partially () and fully () emerged with time in 0.05M Bicine buffer (pH8.5), at 37°C, from eggs aged 2, 5 and 10 days old. For each value, n=5 replicates of 100 eggs each.



5 day old eggs and $2 \pm 0.5\%$ occurred in 10 day old eggs.

The effect of pepsin/HCl and temperature on larval shrinking at different NaCl concentrations.

Materials and methods

Unembryonated faecal eggs were incubated at 21°C for 5 days. Larval shrinking was measured in fifteen to twenty eggs using the timelapse video system (Section 2.1.6) at each of the following NaCl concentrations 0.3, 0.6, 1.2 and 2.5M plus a control of water, under the following conditions i) at 25°C, ii) at 37°C, iii) at 37°C following pretreatment in 0.5% pepsin/0.005M HCl for 1 hour. Larval shrinking was measured hourly using a computer program devised by B.E. Matthews (Univ. of Edinburgh), which allowed two points to be superimposed on the video screen at either end of each larva. The mean \pm SE length of the larvae was then calculated on a BBC microcomputer. The results are presented in Fig.17.


Results

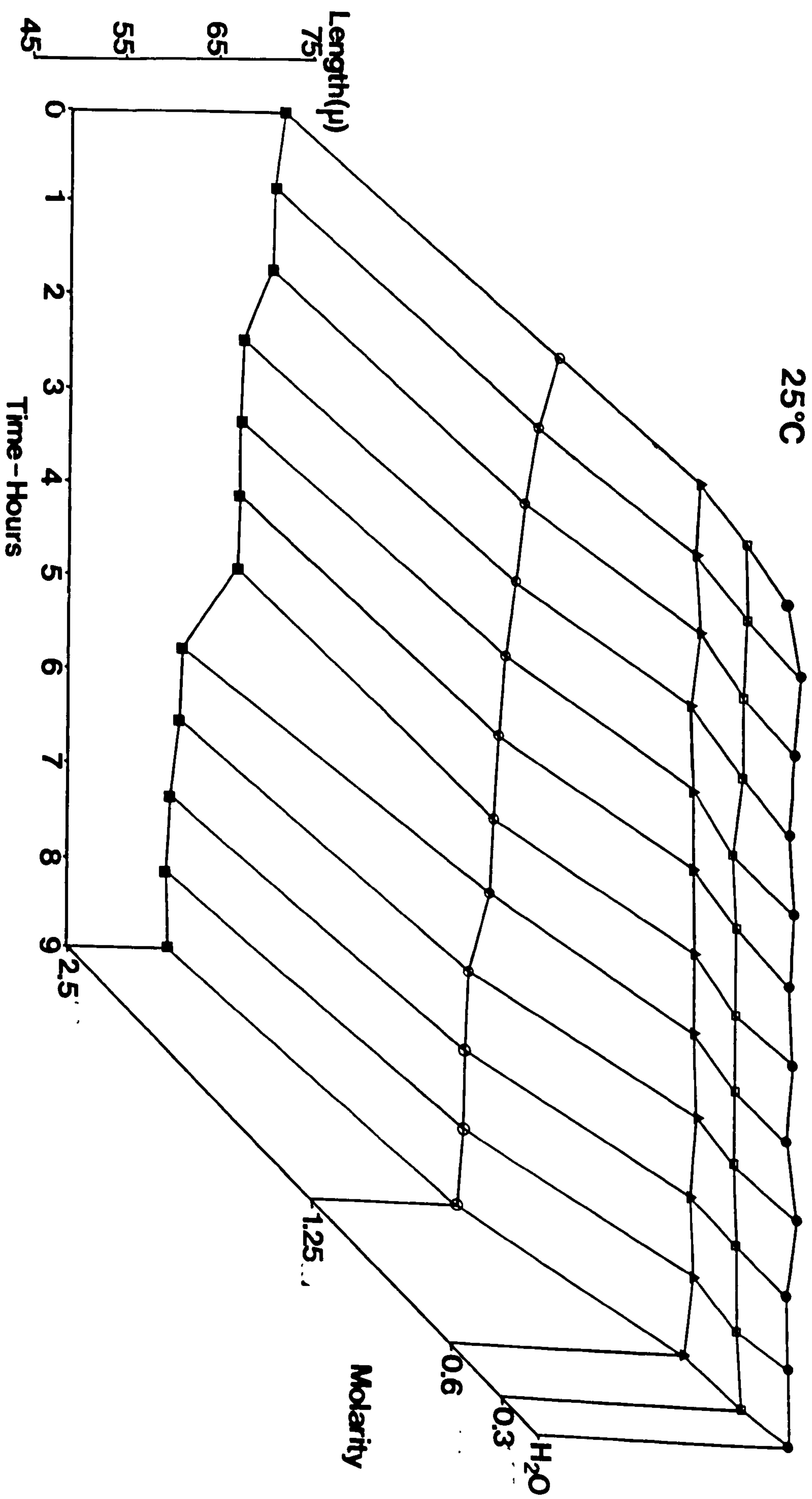
The effect of increasing NaCl concentrations on larval length at 25°C, 37°C and 37°C following pretreatment with pepsin/HCl is illustrated in Fig.17. At equivalent NaCl concentrations, increased levels and rates of shrinking at 37°C occurred in eggs previously incubated in pepsin/HCl. Larval shrinking was much reduced at 25°C. For each treatment, larval shrinking increased with increasing NaCl concentration. No shrinking occurred in water and 0.3M NaCl following any of the treatments. In 0.6M NaCl, no shrinking occurred at 25°C. At 37°C and after incubation in pepsin/HCl, larval length was reduced by about 14% and 30.5%, at a rate of approx. 0.06u and 0.15u per minute respectively. In 1.2M NaCl, a final reduction in length of about 12%, 30% and 42.5% occurred at a rate of approx. 0.02u, 0.075u and 0.2u per minute at 25°C, 37°C and 37°C following treatment with pepsin/HCl. In 2.5M NaCl, larvae decreased in length by about 34.5%, 38% and 43.5% at a rate of approx. 0.03u, 0.3u and 0.35u per minute at 25°C, 37°C and 37°C after incubation in pepsin/HCl respectively.

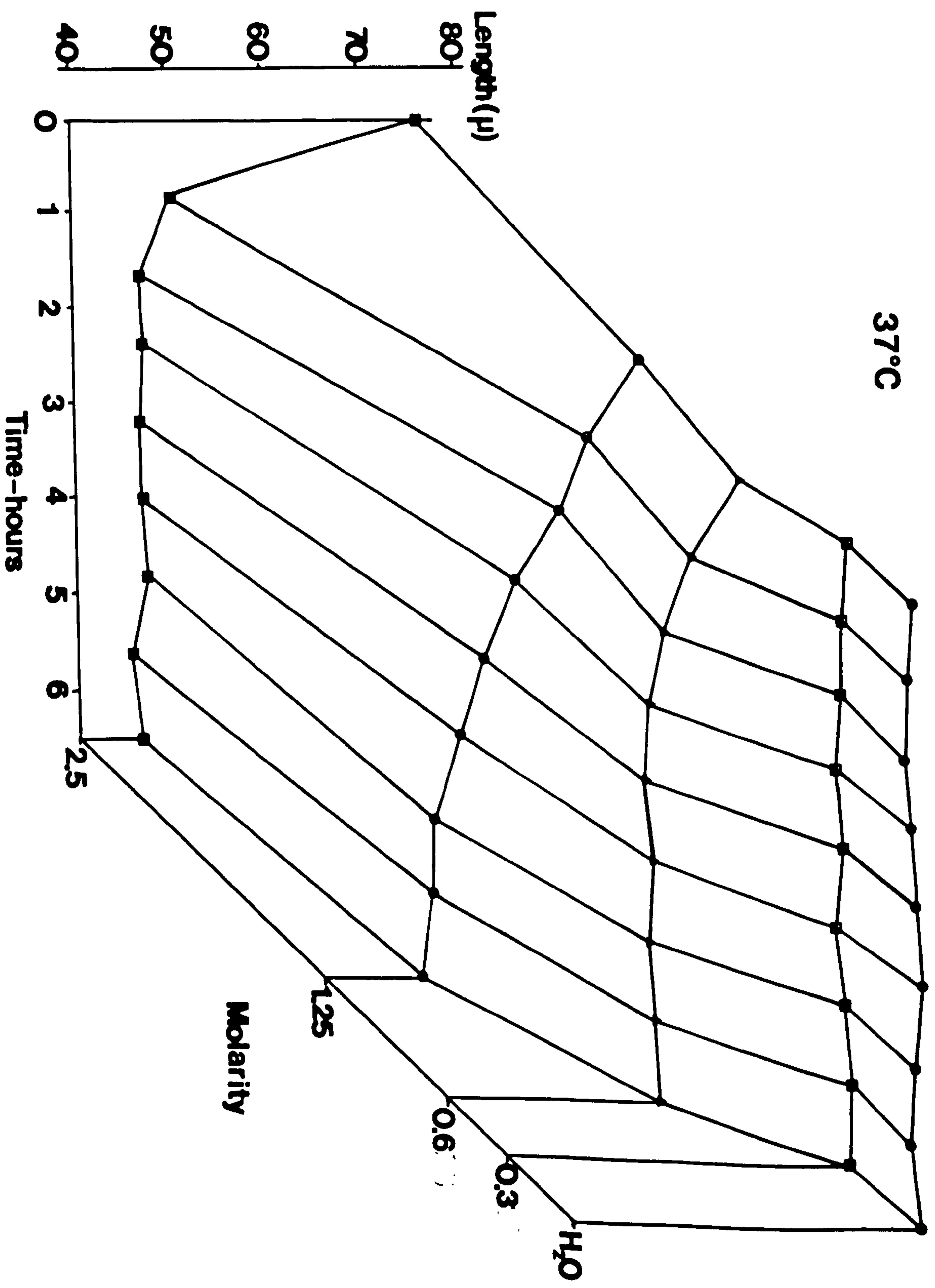
The effect of pepsin-HCl, trypsin and Bicine buffer (pH 8.5) at 21°C and 37°C on egg staining in osmium tetroxide.

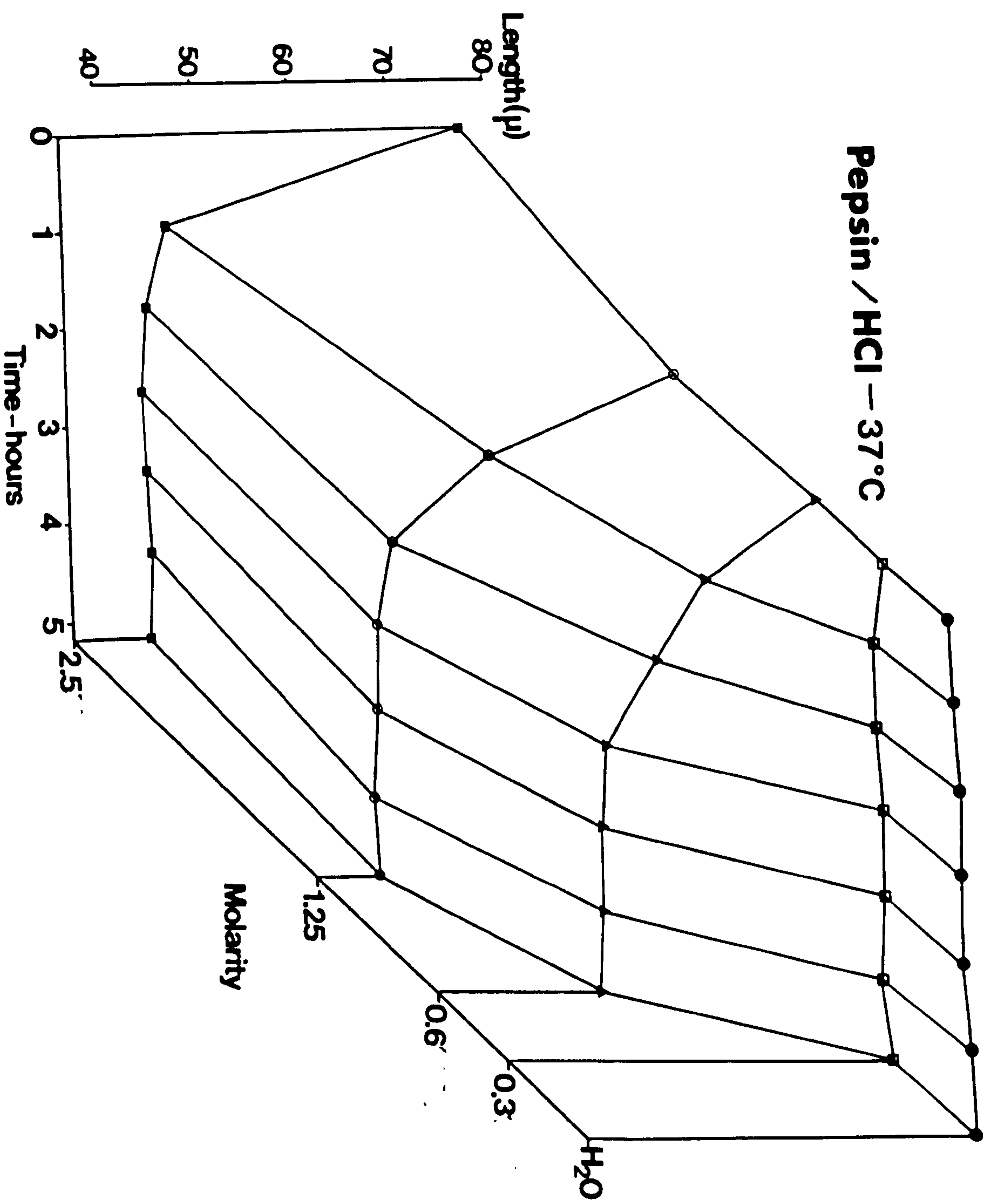
Fig.17.The effect of pepsin-HCl and temperature on larval shrinkage in NaCl solutions.



Mean_±SE percentage shrinkage () with time of eggs incubated in a range of NaCl concentrations (0.3-2.5M) at i) 25°C, ii) 37°C and iii) at 37°C, after pretreatment with pepsin-HCl at 37°C for 0.5hour. For each treatment 15-20 eggs were examined.







Materials and methods

Unembryonated faecal eggs were incubated for 5 days at 21°C. Batches of eggs were then incubated in the following treatments:- i) 0.05M Bicine buffer (pH 8.5) at 21°C and 37°C, ii) 0.005M HCl at 21°C continuously, iii) 0.5% pepsin/0.005M HCl for 4 hours at 37°C prior to incubation in Bicine buffer (pH 8.5) at 37°C, iv) pepsin/HCl at 21°C continuously, v) 0.5% trypsin, for 4 hours at 37°C prior to incubation in Bicine buffer (pH 8.5), at 37°C, vi) trypsin at 21°C continuously, vii) 0.05% dithiothreitol, for 4 hours at 37°C prior to incubation in Bicine buffer (pH 8.5), at 37°C and viii) 0.05% dithiothreitol at 21°C continuously. At regular intervals 2 replicate samples of 100 eggs each were removed and stained with osmium tetroxide in 2.5ml lidded plastic tubes (Sarstedht Ltd.) for 12 hours at 4°C. Egg staining was assessed according to the categories illustrated in Fig.13a,b,c. The results are presented in Fig.18.

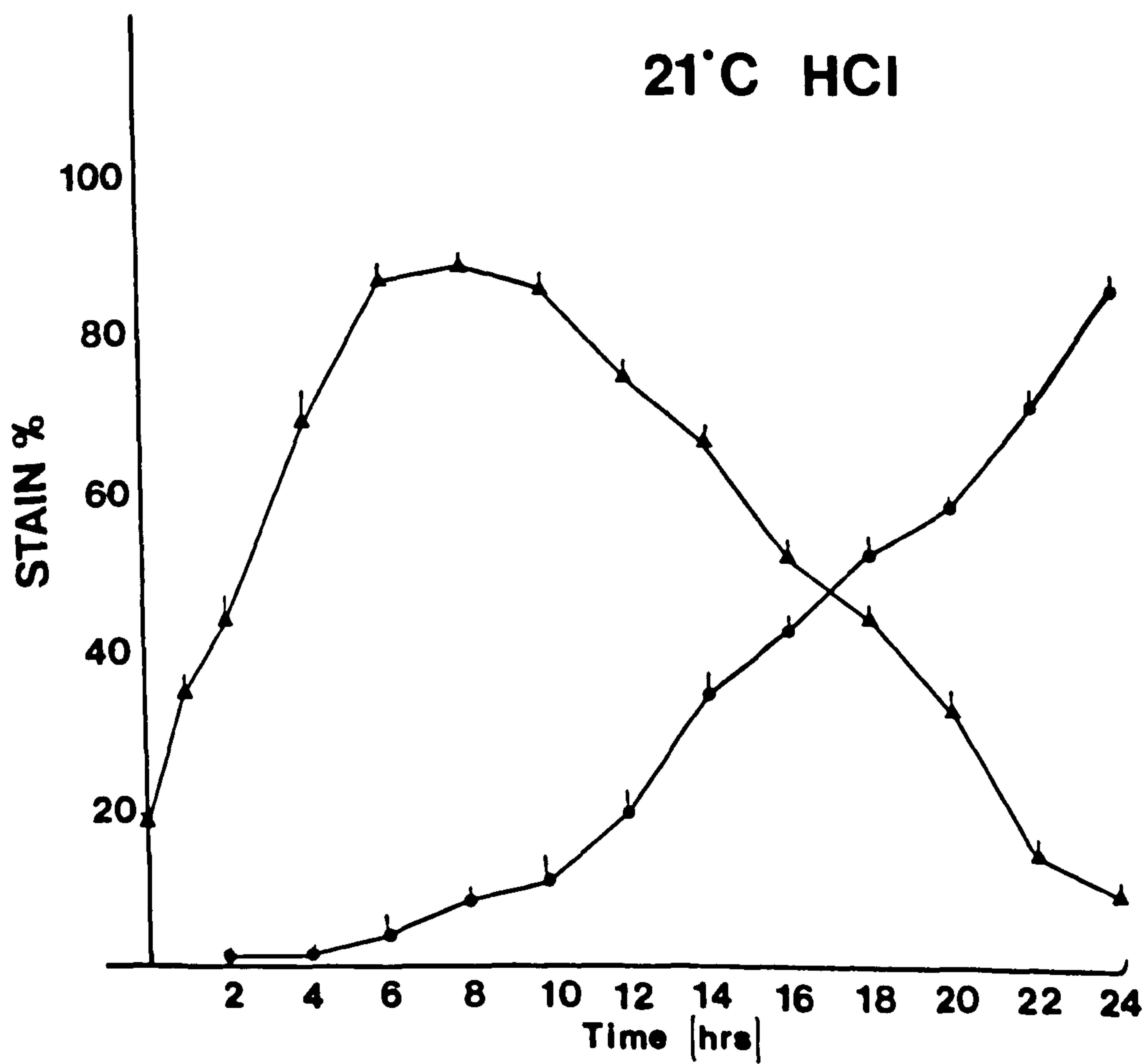
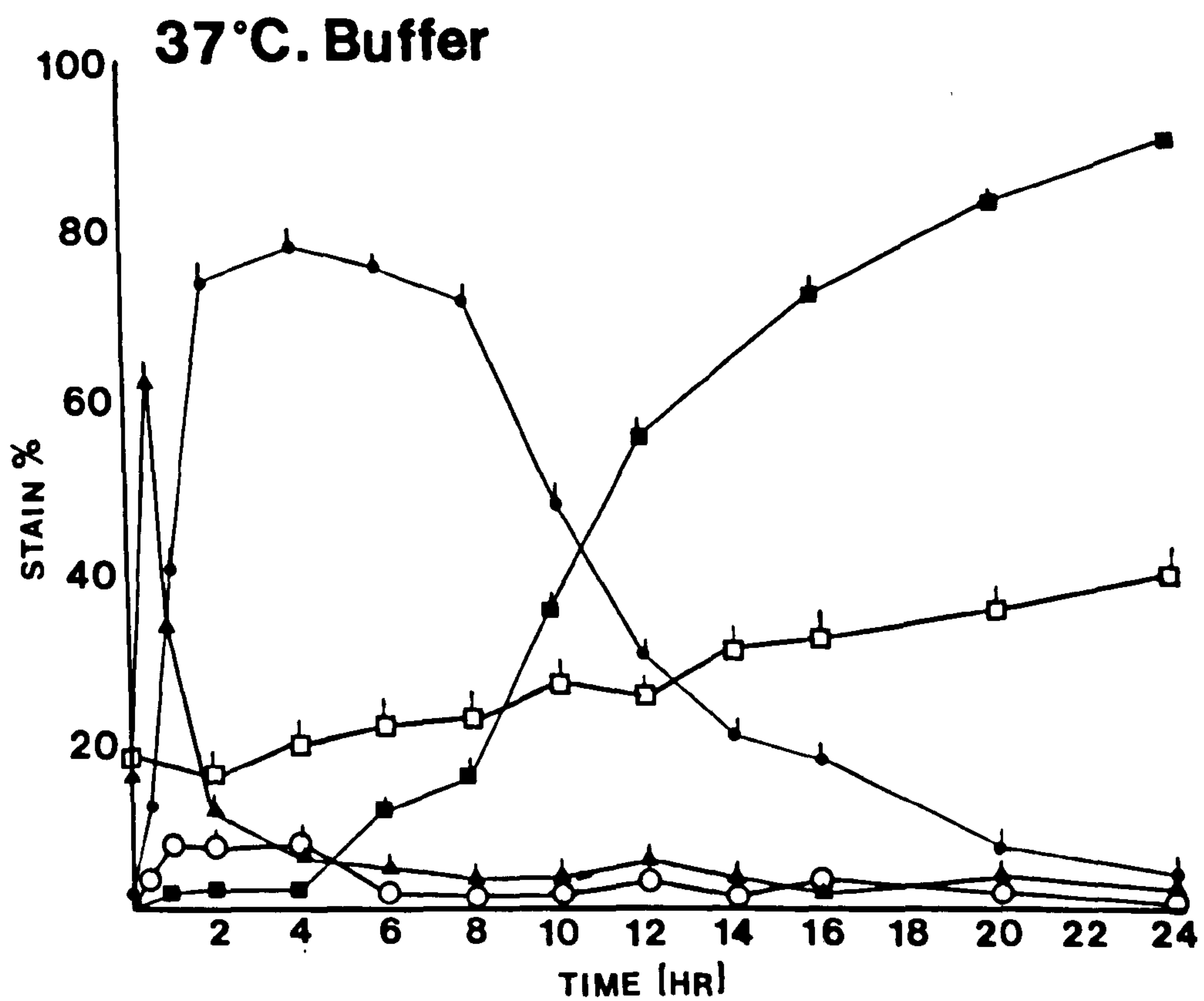
Results

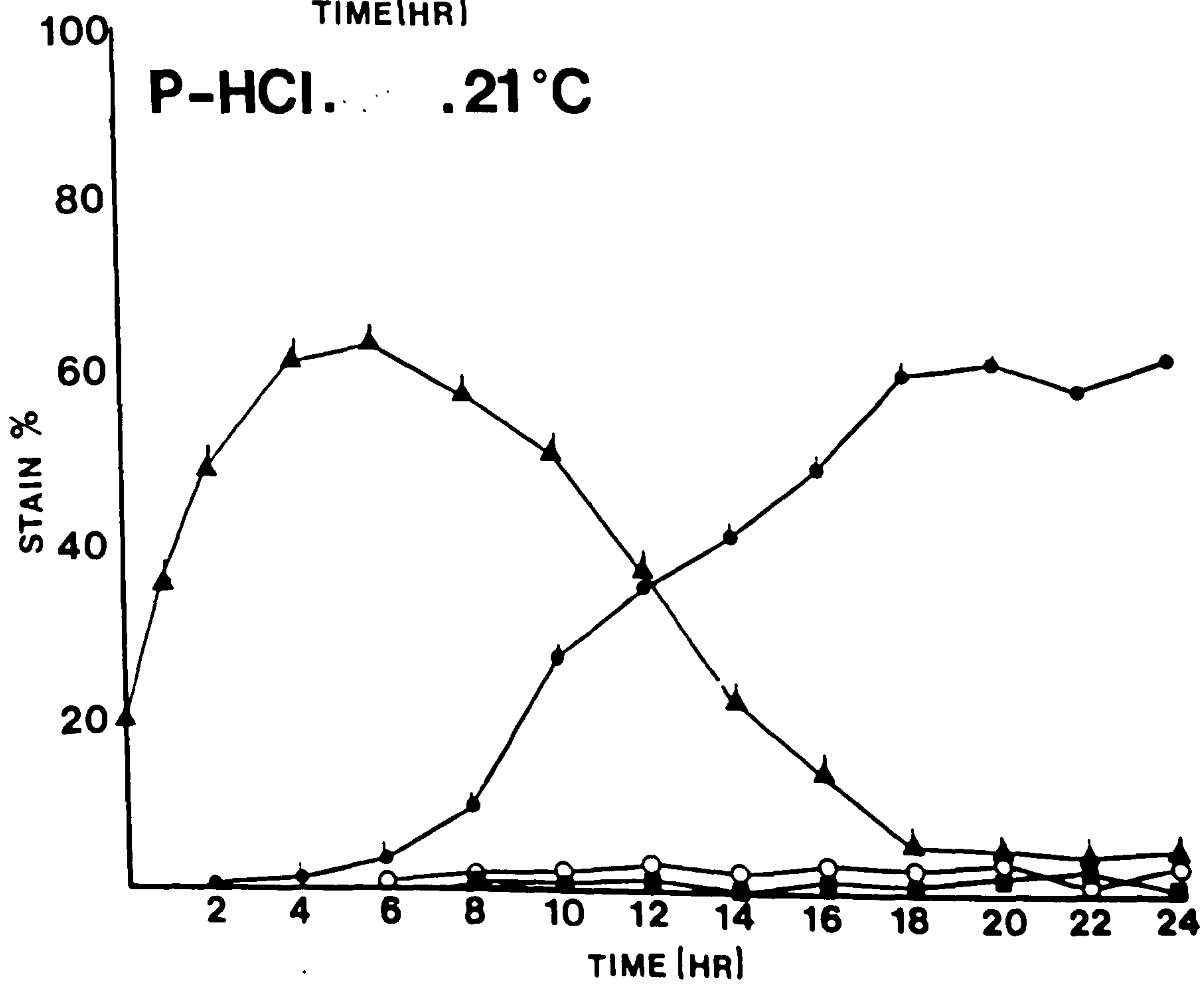
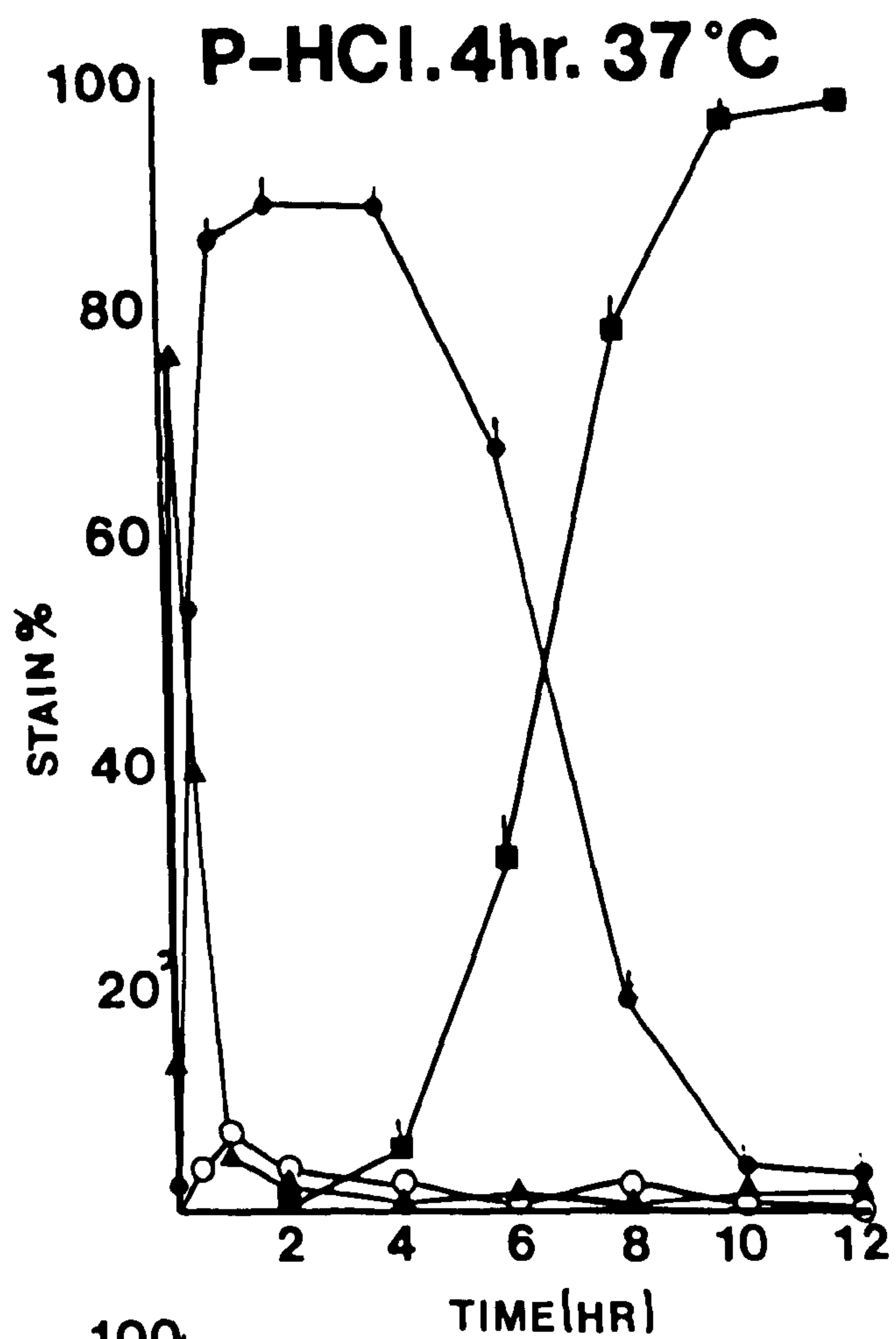
In general the pattern of staining was similar in all treatments (Fig.18.), although the rate of occurrence was dependent on the specific conditions. Initially egg fluid only stained, followed by larval staining within the egg and then emerged larval staining. A decrease in one type of staining corresponded to an increase in the following type of staining. The rate of staining was increased in pepsin/HCl and trypsin at 37°C compared to buffer at 37°C. The rate and level of staining found in any treatment at 21°C was much reduced.

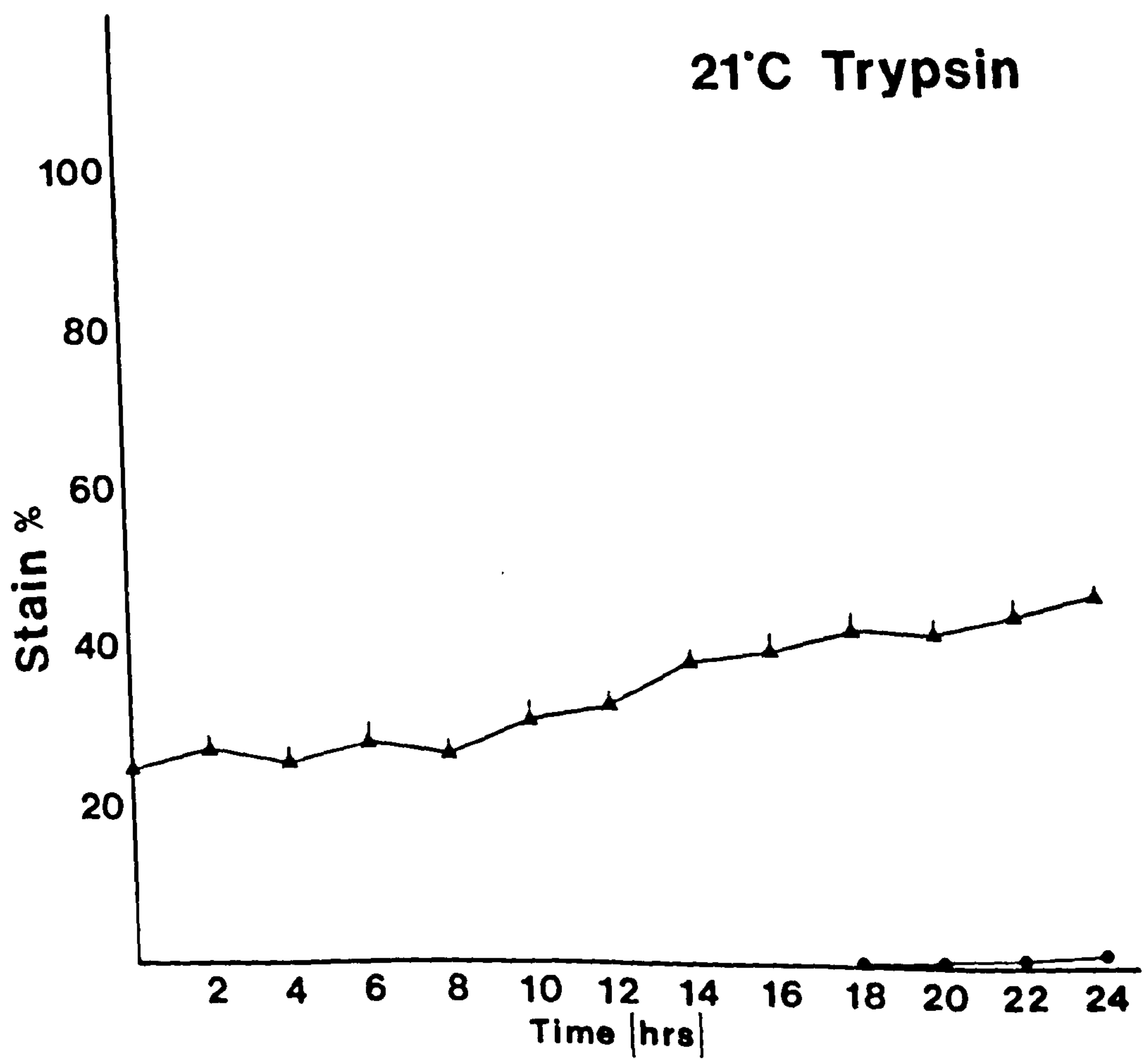
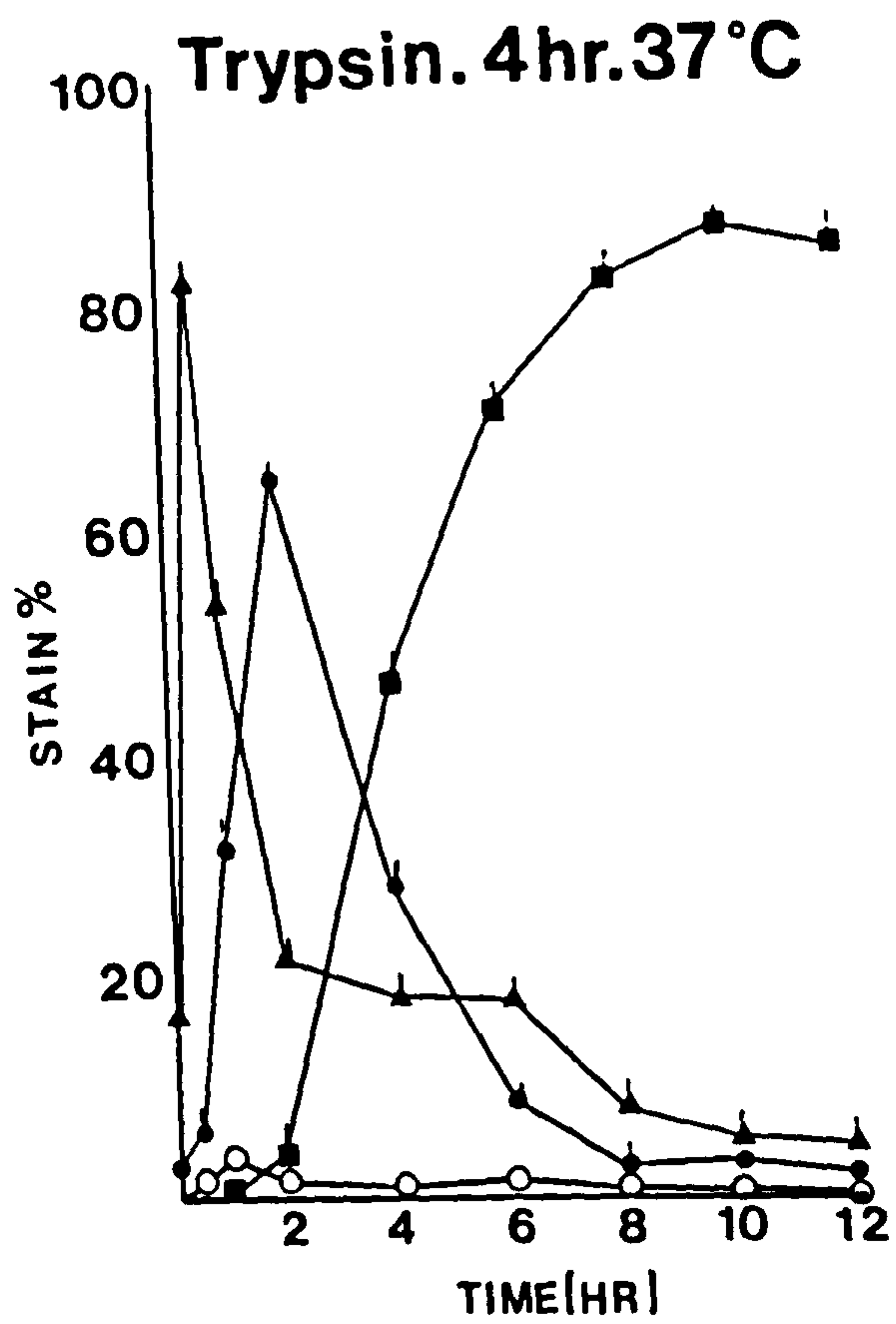
At 21°C, only egg fluid stained in buffer only and the trypsin treatment, increasing from about 20% to 40%. In pepsin/HCl and HCl at 21°C egg fluid staining reached about 70% to 75% after 10 hours incubation and then decreased to between about 20% and 25% after 24 hours. Larval staining in pepsin/HCl reached $60 \pm 4\%$ between 8 and 24 hours incubation and stained emerging larvae reached a maximum level of $3 \pm 1.5\%$ from 10 hours onwards. After incubation for 1 hour in dithiothreitol, egg fluid only staining reached $80 \pm 4.5\%$ and decreased to less than $5 \pm 2\%$ after 10 hours incubation. Larval staining within the egg reached $85 \pm 4\%$ after 8 hours and remained at this level. Between approx. 80% and 90% of the larvae within the eggs which stained at 21°C for all treatments, were found to be stained only at the tip nearest the operculum. Larval emergence was less than $3 \pm 1\%$.

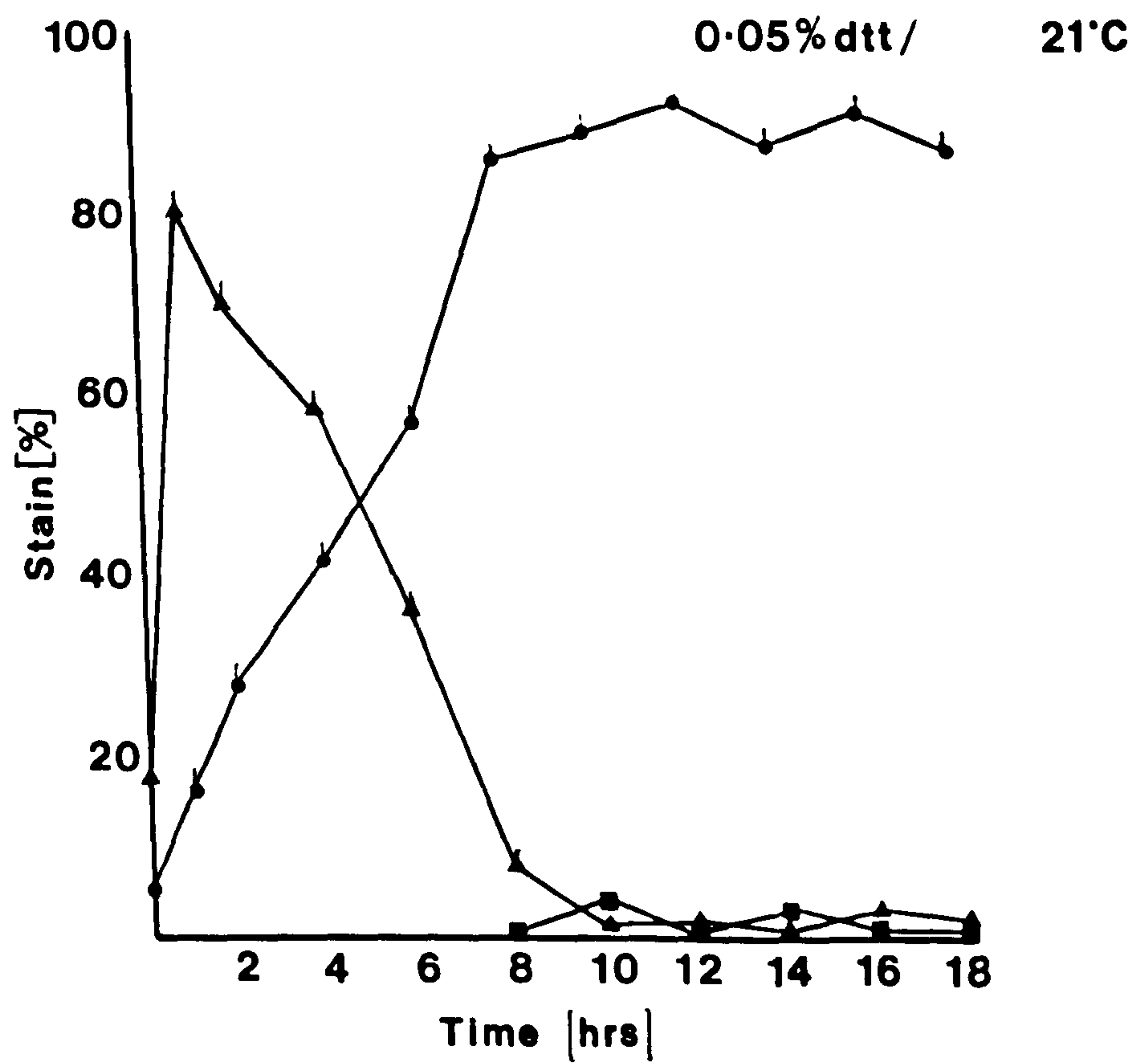
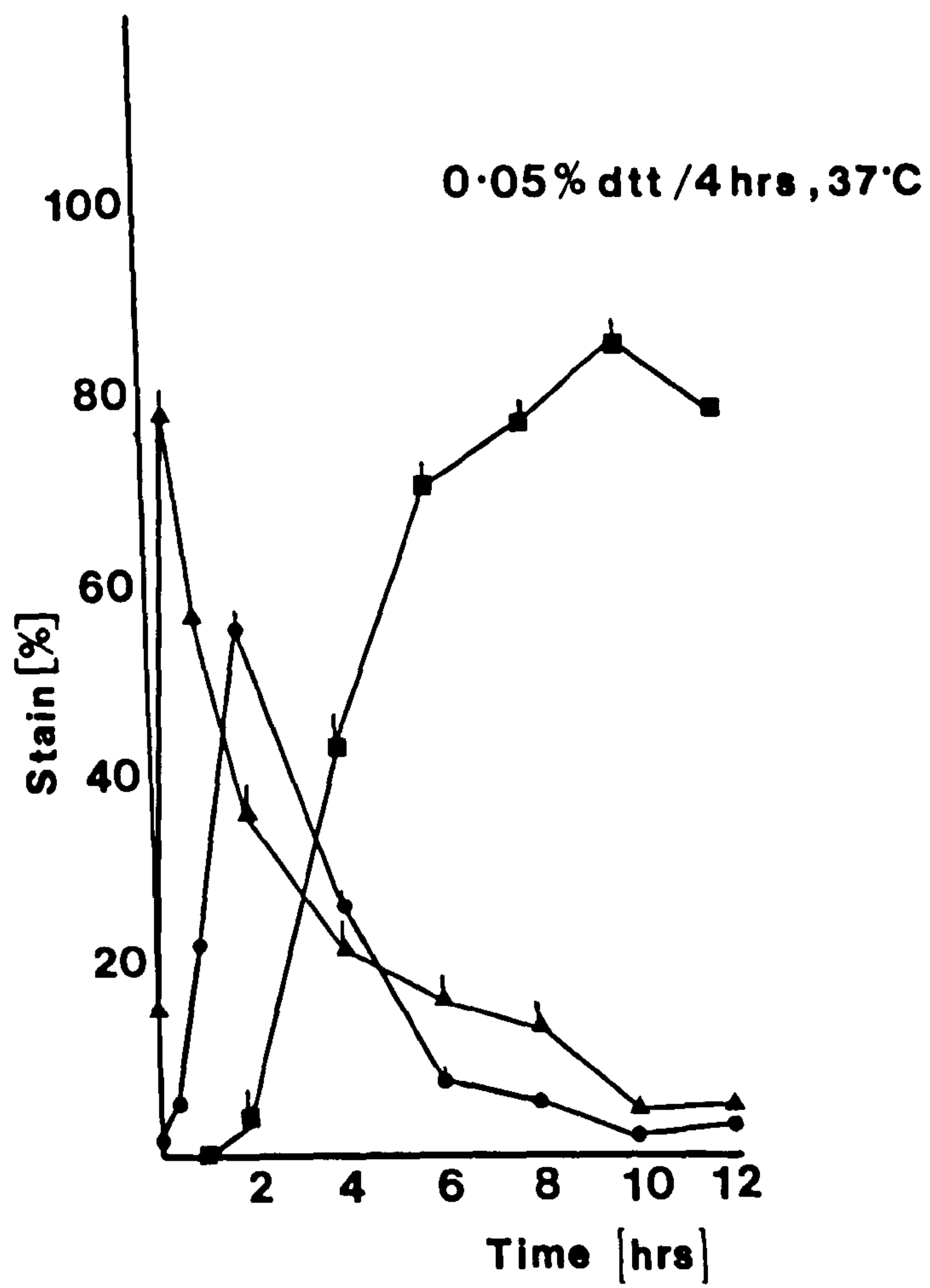
Fig.18. The effect of pepsin-HCl, trypsin and bicine buffer (pH 8.5) at 21°C and 37°C on staining of eggs in OsO₄.

Differential staining of eggs in OsO₄ at regular intervals during the following treatments i) buffer, 37°C continuously, ii) 21°C, HCl, iii) pepsin-HCl, trypsin, dithiothreitol, 4 hours, 37°C, iv) buffer, pepsin-HCl, HCl, trypsin, dithiothreitol, 21°C. Types of staining found included egg fluid only (▲—▲), larval only (●—●), both egg fluid and larval within the egg (○—○) and free larvae (■—■). Egg fluid only at 21°C in buffer (□—□). For each value, n=2 replicates of 100 eggs each.









In buffer at 37°C, egg fluid staining increased by about 50% in 30 minutes reaching $63 \pm 4.5\%$. It then decreased to between about 5 and 10% after 4 hours incubation. Larval staining reached levels of between approx. 75 and 80%, 2 to 4 hours after incubation and then decreased to less than $5 \pm 2\%$ by the end of the experiment. Stained emerging larvae increased from $2 \pm 0.5\%$ to $85 \pm 3\%$ between 4 and 24 hours incubation.

In pepsin/HCl, trypsin and dithiothreitol, all at 37°C, egg fluid staining reached $75 \pm 3.5\%$, $82 \pm 3\%$ and $78 \pm 4\%$ after 15, 30 and 30 minutes respectively. It then rapidly decreased to less than about 5% after a further 45 minutes in pepsin/HCl, 9 hours in the trypsin treatment and 10 hours in the dithiothreitol treatment. Larval staining within the egg reached $85 \pm 3.5\%$, $65 \pm 3\%$ and $56 \pm 3\%$ after 1, 2 and 2 hours incubation in the pepsin/HCl, trypsin and dithiothreitol treatments respectively, thereafter decreasing to less than about 5% after 8, 10 and 10 hours incubation respectively. After stimulation by pepsin/HCl, hatching reached $95 \pm 3\%$ after 8 hours by trypsin $87 \pm 2.5\%$ after 10 hours incubation and by dithiothreitol $85 \pm 4.5\%$ after 10 hours incubation.

In all treatments, the level of staining found for egg fluid and larva combined remained constant at levels less than approx. 5% for the duration of the experiment. General observations showed that larval staining was initiated in the region closest to the operculum, in eggs incubated in pepsin/HCl, dithiothreitol and HCl, at 21°C.

Changes in the eggshell during hatching and in pepsin/HCl, trypsin and dithiothreitol at 37°C.

Materials and methods

Unembryonated faecal eggs were incubated at 21°C for 5 days. For each of the following treatments, batches of eggs (approx. 500 eggs in each) were incubated in 0.5ml of the solutions in 3 ml lidded plastic testubes (Sarstedt Ltd.):— i) 0.05M Bicine buffer (pH 8.5) at 37°C for 1, 2, 4, 8, and 12 hours, ii) 0.5% pepsin/HCl at 37°C for 0.5, 1, 2 and 4 hours, iii) 1% trypsin in 0.05M Bicine buffer (pH 8.5) for 0.5, 1, 2 and 4 hours, iv) 0.05% dithiothreitol in 0.05M Bicine buffer (pH 8.5) for 0.5, 1, 2 and 4 hours and v) 0.05M Bicine buffer (pH 8.5) at 21°C for 12 hours (control). Following each treatment the eggs were rinsed twice in distilled water and prepared for transmission electron

microscopy as described in Section 2.1.5. For each treatment another batch of eggs was incubated until hatching occurred and was prepared for low temperature scanning electron microscopy also detailed in Section 2.1.5.

Results

The impermeability of unstimulated eggs at 21°C to penetration by fixatives prevented transverse sections of whole eggs being obtained. Sections of the egg wall show that many eggs have undisrupted layers (Plate 10a), but in some eggs the uterine layers have begun to disintegrate and stained particulate material is present in close proximity to the lipid layer (Plate 10b). At 21°C, all the opercula observed were intact (Plate 11a).

Sections of hatching eggs obtained from eggs stimulated in buffer, pepsin/HCl, trypsin and dithiothreitol at 37°C, indicated that the sequence of changes in the eggshell during hatching was similar in all the treatments but occurred at different rates.

After incubation for between 1 to 2 hours in buffer, 0.5 to 1 hour in trypsin, 0.5 to 1 hour in dithiothreitol and 0.25 to 0.5 hour in pepsin/HCl, the egg layers of the shell began to separate (Plate 11b). The uterine layers began to disintegrate (Plate 12a) and an increase in particulate material along the inner edge of the lipid layer was observed. In many eggs the vitelline layer was separating from the internal uterine layer (Plate 12b), but the operculum remained intact (Plate 13a). Stimulation by pepsin/HCl and trypsin made the eggshells soften and prone to collapse (Plate 13b).

Increasing incubation in all treatments, between 4 and 8 hours in buffer, 2 and 4 hours in trypsin, 2 and 4 hours in dithiothreitol and 1 and 2 hours in pepsin/HCl led to an increase in particulate material within the egg (Plate 14a) and disintegration of the operculum (Plate 14b). In eggs where the lipid layer had separated from the chitin layer, a membrane was observed enclosing the larvae (Plate 15b). In some sections it was observed that the operculate end of the egg had collapsed trapping the larva within the egg (Plate 15a & 16a). In the section shown in Plate 16b, one section of the lipid layer is adjoined to the chitin layer and the other section is attached to the larva. Many of the larvae which emerged from the eggs, after 8 hours in buffer, 4 hours in trypsin and dithiothreitol and 2 hours in pepsin/HCl, were still enclosed in a

membrane (Plate 17a & 17b). Larvae emerged through the operculum in all the eggs observed (Plate 18a). The collapsed inner layers of the hatched eggshell are shown in cross-section in Plate 18b.

Scanning electron micrographs of emerging larvae and the exterior of empty eggs are given in Plates 19a & b and 20a & b.

Enzyme assay

Materials and method

To obtain large quantities of eggs, at least 30,000, the following mass extraction technique was used. Faeces were emulsified in tap water, passed through a 1 mm sieve and rewashed with tap water. The faecal debris was discarded and the filtrate allowed to sediment for at least 1 hour, before the supernatant was removed by suction. The sediment was centrifuged in 100ml plastic centrifuge tubes for 2 minutes at 1,500 r.p.m. The supernatant was again discarded and the remaining pellet of faecal debris containing the eggs resuspended in saturated salt solution and centrifuged for a further 2 minutes at 1,500 r.p.m. The top layer of salt solution containing the floating eggs was dispensed into another centrifuge tube and the eggs washed twice with distilled water.

Following incubation for 5 days at 21°C, the eggs were washed twice in distilled water and twice in penicillin/streptomycin solution (Sigma Ltd., working concentration, 1mg/100ml). The last washing was used as a control and contained in a 5ml bijou bottle (B.D.H.Ltd.). The eggs were resuspended in 3ml 0.05M Bicine buffer (pH 8.5) also in a bijou bottle. Azocoll (10mg/ml), (Calbiochem. Ltd.) was added to both the control and egg sample. At periodic intervals, the supernatants from the control and egg sample was removed and centrifuged to concentrate any particulate matter. The two debris free supernatants were each in turn placed in 2ml clean cuvettes and the light absorption of the solutions at 520nm was measured spectrophotometrically against a distilled water blank. The supernatants were then returned to their respective solutions and reincubated at 37°C.

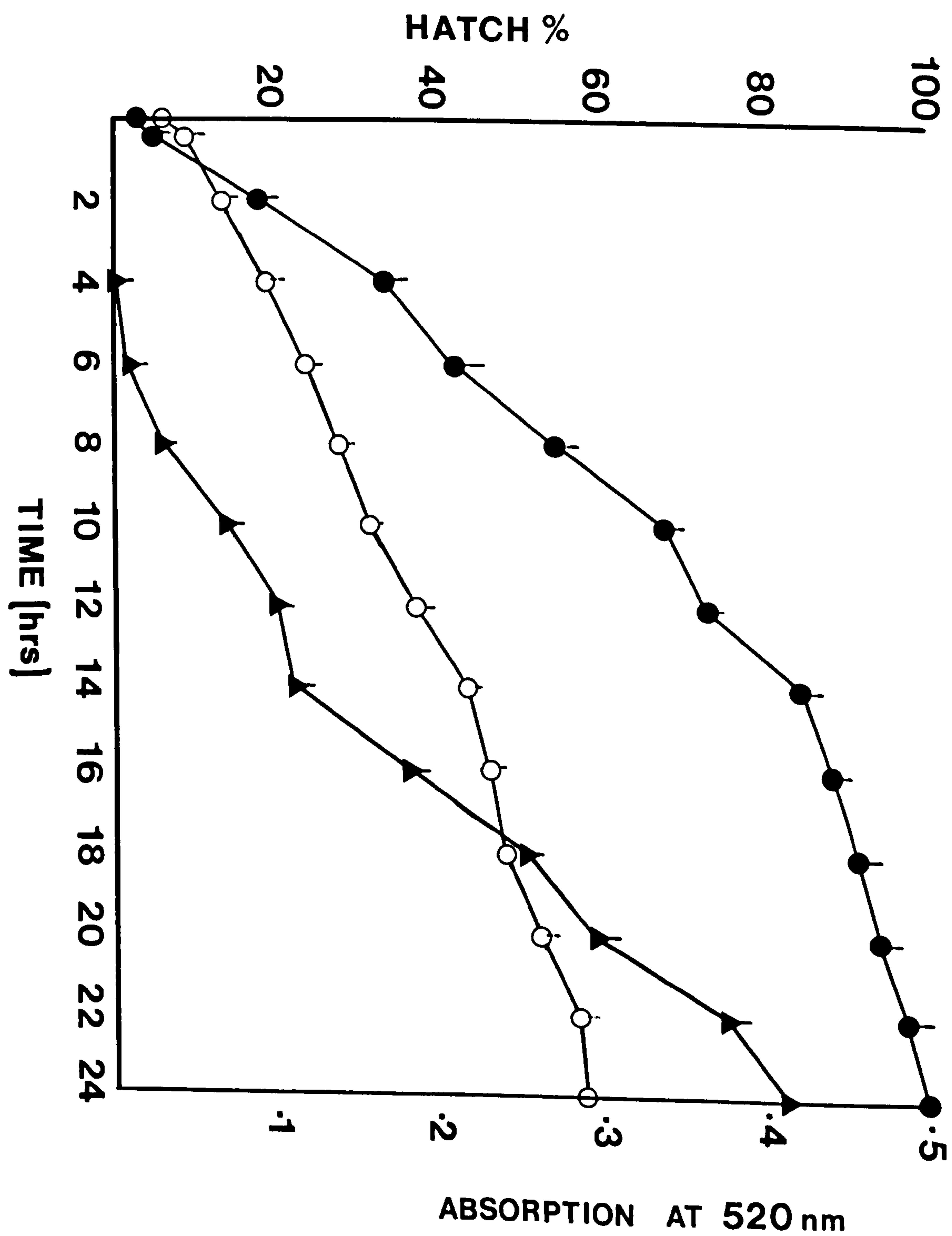
To assess the rate of hatching of the eggs, 3 replicates of 0.01ml of egg suspension was removed at regular intervals and the percentage hatch of 100

eggs calculated as described in Section 2.1.4. The results of the enzyme assay and hatching are presented in Fig.19.

Fig.19.Enzyme assay.

i) Mean \pm SE percentage hatch of eggs ($\blacktriangle\text{---}\blacktriangle$) at regular intervals incubated in 0.05M Bicine buffer (pH 8.5), at 37°C. For each value, n=3 replicates of 100 eggs each.

ii) Absorption of light at 520nm by hatching medium ($\bullet\text{---}\bullet$) and the control ($\circ\text{---}\circ$).



Results

The absorption of light (520nm) for the control and egg suspension over the course of the experiment and the rate of hatch obtained are presented in Fig.19. An increase in absorption of light occurred in the egg suspension (0.02 to 0.5) compared to the control (0.01 to 0.29), however the proportional rate of increase in both treatments indicates that bacterial/fungicidal contamination has occurred. The difference obtained in absorption between the two samples is likely to be due to increased levels of contamination in the egg suspension. A level of hatch of $83 \pm 2\%$ occurred between 4 and 24 hours incubation at 37°C .

Larval activity at 21°C and 37°C .

Materials and Methods

Unembryonated eggs were extracted from 2 hour old faecal samples. The time lapse video system, described in Section 2.1.6 was used to record larval activity at 21°C and 37°C . Eggs were incubated in 0.05M Bicine buffer (pH 8.5) at 21°C . Once the larvae were capable of rotating on their own axis, the eggs were continuously recorded in 80 hour mode (1 frame/1.4 secs.). Larval activity was recorded for 3 minutes every hour at "normal speed" (1 frame/0.02secs.) using the alarm facility. Larval activity was analysed via an event recorder which enabled the activity of 8 eggs to be monitored simultaneously, linked to a BBC microcomputer using a program devised by Matthews (1985). The mean \pm SE percentage time active for the larvae was calculated. The results are presented in Fig.20.

Results

The mean \pm SE percentage time active for larvae at 21°C and 37°C is illustrated in Fig.20. At 21°C , activity remained relatively constant around 10%. At 37°C , activity increased to between about 65 and 75% after 4 to 8 hours incubation. From 8 hours onwards, activity decreased, to a level of approx. 20% after 24 hours incubation at 37°C . No hatching occurred at 37°C .

General observations on hatching behaviour

Materials and methods

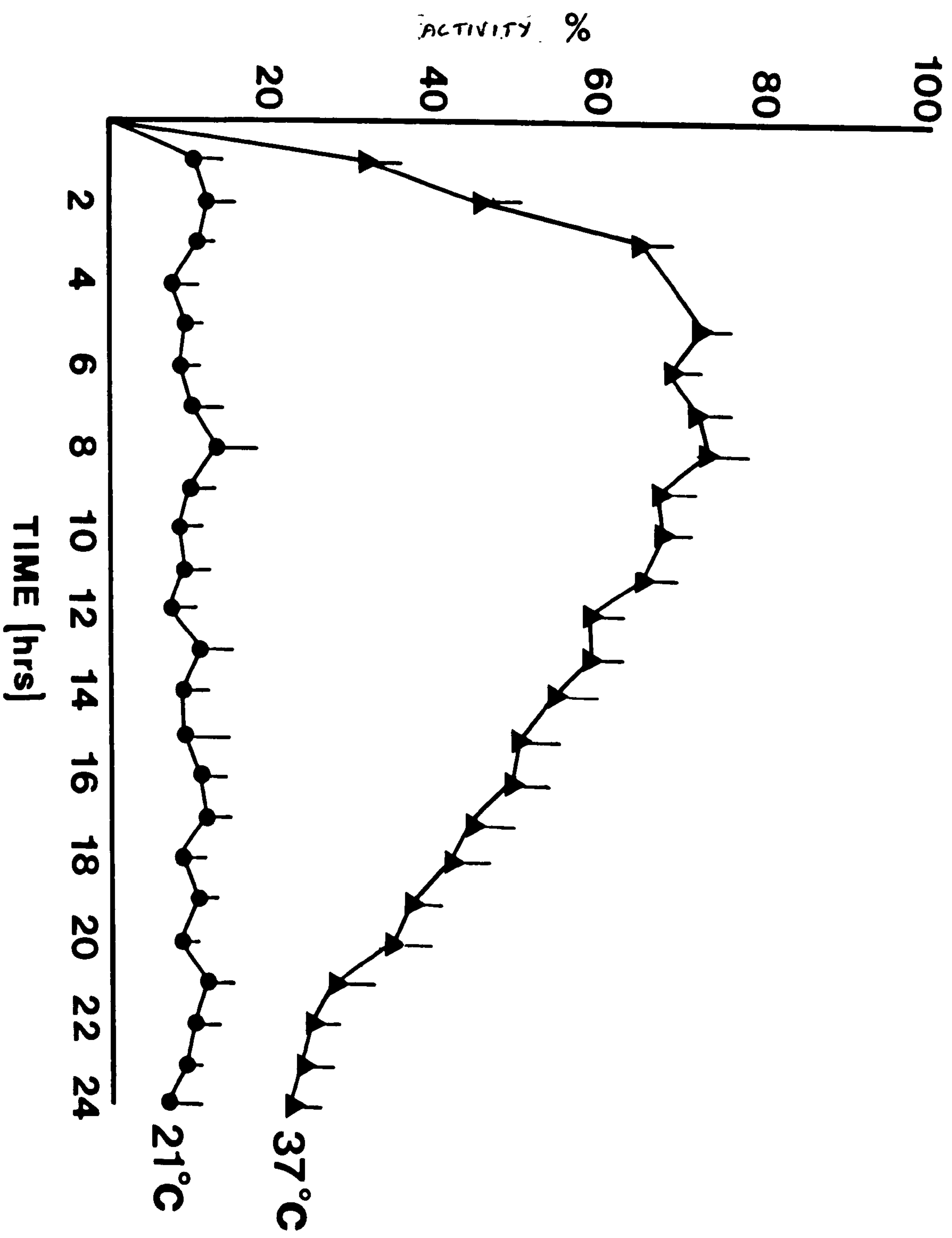


Fig.20.The effect of temperature on larval activity.

Mean±SE percentage time active within the egg at 21°C (●—●)and 37°C (▲—▲), n=8 eggs.

Unembryonated eggs were extracted from 2 hour old faecal samples and incubated at 21°C for 5 days. Larval behaviour and hatching was examined in 15 to 20 eggs using the timelapse video system described in Section 1.6., during incubation in pepsin/HCl at 37°C and in bicine buffer (pH 8.5) at 37°C, after pretreatment in pepsin/ HCl for 1 hour. Photographs were taken of larvae hatching at regular after stimulation by pepsin/HCl.

Results

During incubation in pepsin/HCl, the larvae appeared suddenly^{to} burst out of the egg, emerging very rapidly as if a pressure release had occurred. In eggs, briefly incubated in pepsin/HCl prior to incubation in buffer at 37°C, 2 of the eggs emerged in the pepsin/HCl as described above, the others hatched in the buffer as follows. They increased their activity with the larvae rotating on their axis inside the egg and occasionally turning around. At least two of the layers of the eggshell were seen to seperated from the uterine layers (Plate 21a) and collapse around the larvae (Plate 21b). Contact appeared to be made with the operculum by random movements, eventually the larvae pushed through the operculum (Plate 21b). In all the eggs observed the larvae emerged head first. They pushed themselves out of the eggshell using their tails as a lever against the inner wall of the eggs. The time taken for the larvae to emerge from the egg varied between 5 and 25 minutes. In some of the empty eggshells the collapsed membranes remained visible (Plate 22a), in others the hatched larvae appeared to emerge still enclosed in the egg membranes (Plate 22b).

4.3.3 Discussion

Permeability changes were demonstrated in the eggs of *A.tetraptera* of increasing age and during stimulated hatching by observing the effect of NaCl solutions on larval length and hatching ability, and the pattern of egg staining in osmium tetroxide. The permeability changes which occurred in eggs which spontaneously hatched during developmental incubation and in eggs stimulated to hatch were similar, although the rate of occurrence varied depending on the conditions to which the eggs were exposed. The eggshells of these eggs were examined by transmission and scanning electron microscopy. Evidence is presented below of the sequence of permeability changes which occur in the eggs and their effect on the eggshell.

The permeability of the egg to water increased with age as demonstrated by i) the development of larval shrinking in eggs incubated in 1M NaCl for 48 hours at 25°C and ii) the increased reduction in hatching levels of eggs previously incubated in concentrations of between 0.25 and 1M NaCl for increasing lengths of time. The higher the concentration of the incubation solution, the shorter the period of incubation prior to a deleterious effect on hatching and the lower the number of larvae which emerged. These observations suggest that NaCl had an osmotic effect on the eggs, the rate and extent of water loss from the eggs being dependent on the concentration and length of exposure of the eggs to the solution.

The differential staining of eggs in osmium tetroxide was used to demonstrate the sequence of permeability changes which occurred in the eggs with increasing age and developmental temperature. Osmium tetroxide was found to be a slow penetrating stain, with increased numbers of eggs staining after increased incubation in the stain for between 1 and 12 hours at 4°C. The black staining of the eggs was more defined after increased incubation in the stain. The pattern of staining which occurred was found to be proportionately similar for eggs of equivalent ages incubated for different lengths of time in the stain. Therefore in successive experiments, eggs were stained for 12 hours in osmium tetroxide, in order to obtain optimum levels and clarity of stain.

Initially, there was an increase in the number of eggs with egg fluid only staining, that is the staining of the egg contents excluding the larva, with

increasing age of eggs, incubated at 21°C. It was demonstrated that the egg fluid stained, as when unstained larvae were pressed from these eggs, a blackened stream of liquid emerged from the operculum. This was followed by an increase in the number of larvae within the eggs staining black, which itself corresponded with a decrease in the number of egg fluid only stained eggs. Finally, a decrease in the number of stained larvae within the eggs was due to larval emergence from the egg and resulted in the presence of free stained larvae.

Osmium tetroxide reacts with lipoprotein complexes which make up most membranes (Dreher *et al*, 1967). It has been reported that as the protein percentage increases in the lipoprotein molecules, the osmium uptake correspondingly decreases (Hayes *et al*, 1963). As saturated fatty acids are not altered chemically by osmium tetroxide, it is most likely that unsaturated fatty acids are involved in the fixation process (Hayat, 1981). He found that procedures which oxidised the double bonds in the lipid bilayer, prior to exposure to osmium tetroxide, blocked the initial staining reaction with osmium tetroxide. Protein bound sulphydryl groups are stained by osmium tetroxide (Wigglesworth, 1964), but proteins are stained weakly or not at all, the stain if any, binding to protein bound lipids (Adams *et al*, 1967 and Adams & Bayliss, 1968). Available evidence indicates that osmium tetroxide does not interact with the pentose or hexose sugars or their polymers (Hayat, 1981).

It is likely therefore that osmium tetroxide is staining lipids in the egg. It appears that the development of staining of the egg fluid contents is due to the increased presence of lipids which may indicate a breakdown of the lipid containing layers of the eggshell. In an attempt to confirm that lipids were being stained, eggs of increasing age were stained in Sudan B Black. The pattern of staining obtained differed from that found in osmium tetroxide. Staining occurring in less than 5% of the eggs, aged 9 days and over and then only the larvae stained lightly. These results also contradict those found by Anya (1964b) and Wharton (1979a), who found the vitelline, uterine layers and the lipid layer gave positive reactions for lipids and phospholipids using Sudan B Black. A likely explanation for these differences is the staining techniques employed. Normal preparations, as used by Wharton (1979a) include sectioning, fixing and alcohol dehydration of the tissue prior to staining with Sudan B Black. In the preliminary experiment conducted in this section, whole, unfixed

eggs were incubated in Sudan B Black. It is probable that the majority of eggs were impermeable to the stain and in those that were not, the increased permeability had resulted in the release of the eggfluid from the egg prior to staining. Unfortunately lack of time precluded further investigations concerning egg staining in Sudan B Black.

The disappearance of egg fluid only staining from eggs of increasing age and the commencement of larval staining indicated further changes were occurring to the eggs. The cessation of egg fluid only staining may be due to either of two causes:- i) a change in the composition of the egg fluid, such that staining no longer occurs i.e., lipids are absent from the contents or ii) a release of the egg fluid from the egg, thus curtailing any further staining of egg fluid by osmium tetroxide. The following observations suggest that the second view is more correct. Firstly, the corresponding increase in larval staining in the egg, initially in the region of the operculum indicates an increase in the permeability of the egg, which would allow release of the egg fluid. Secondly, collapse of the egg membranes within the egg, suggested a decrease in the osmotic pressure of the internal egg environment had occurred. Unstained larvae were active in eggs in which the surrounding egg fluid was stained, however activity ceased once the larvae stained, which suggests that larvae within the eggs were initially protected from osmium tetroxide in some manner. Unstained larvae pressed from egg fluid only stained eggs and incubated in osmium tetroxide, rapidly became blackened and moribund, indicating that larval protection from the stain involves the eggshell, probably the lipid layer (discussed below), as opposed to the larval cuticle.

Obtaining transmission electron micrographs of ageing eggs without disrupting the eggshell wall proved to be difficult due to the impermeability of unstimulated immature eggs to fixation. Wharton (personal communication) obtained sections by squashing the eggs to allow entry of the fixative through the operculum, however this was impractical in this study as undisturbed eggshell walls and opercula were required for observation. From the few micrographs obtained, after prolonged fixation of the eggs (Plates 1 to 10), it was apparent that the eggshell structure altered with the increasing age of the egg. As the eggs aged, the internal uterine layer disrupted followed by separation of the underlying vitelline and/or chitin and lipid layers, which collapsed inwards leaving a space. The lipid layer appeared to remain intact

and in close proximity to the larva. As the eggs aged disintegration of the operculum occurred and stained larvae were observed.

From these observations, it is suggested that the egg fluid only stain occurs in the spaces of the internal uterine layer and the space formed by the separating layers. An increase in the number of eggs staining with age is due to the disintegration of these layers, resulting in lipid staining. Although the accumulation of particulate material in close proximity to the lipid layer indicates some change in this layer, it remains impermeable to osmium tetroxide leaving the larva unstained. The operculum appeared intact at this stage. The inward collapse observed in the chitin and lipid layers, the decrease in egg fluid staining and corresponding increase in larval staining suggest a change in the permeability of the egg, resulting in a release of the egg fluid contents and the entry of osmium tetroxide, either via the egg wall or the operculate region. The apparently intact operculum in eggs with collapsed eggshell layers suggests that the egg fluid may leave the egg via the pores of the external uterine layer. The development of larval staining may be a result of a further change in the permeability of the lipid layer allowing the osmium tetroxide to reach the larva and/or a change in the permeability of the operculum. Staining of the egg contents of non viable eggs suggests that the lipid layer was impaired, this may be the reason for their non-development.

An increase in the developmental temperature of eggs resulted in an increase in the rate of occurrence of the different types of staining although the sequence of staining remained the same. This increase in the development of permeability with temperature was associated with an increase in the number of free stained larvae. Therefore the increase in spontaneous hatching with increased developmental temperature, as demonstrated in Section 3.2., is a direct result of the permeability changes described above.

The increased rate of hatching observed in ageing eggs, once stimulated by incubation in buffer at 37°C, suggests that the changes in permeability demonstrated in the egg with age represent the initial processes of the hatching of eggs.

In eggs stimulated to hatch in various treatments such as buffer, pepsin/HCl, trypsin and dithiothreitol, all at 37°C, the same permeability changes which occurred during spontaneous hatching of ageing eggs were

observed, but their rate of occurrence was increased. An increased rate of larval shrinking occurred in eggs incubated in concentrated NaCl solutions after stimulation in pepsin/HCl at 37°C, compared to 37°C only. The rate of staining of eggs in osmium tetroxide also increased after stimulation in pepsin/HCl, trypsin and dithiothreitol, compared to buffer, all at 37°C. Staining was most rapid after stimulation in pepsin/HCl. Trypsin and dithiothreitol produced similar rates of staining. At 21°C, the rate of staining was much reduced for HCl and dithiothreitol. Pepsin, trypsin and buffer had no effect on staining at 21°C. These results show that the enzymes, pepsin and trypsin only affect the permeability of the eggshell at 37°C, the temperature at which the enzymes might be expected to work *in vivo*. Dithiothreitol and HCl, alter the permeability of the eggshell regardless of the temperature. The development of larval staining in eggs incubated under these two conditions, in the region nearest the operculum suggest that they directly affect the permeability of the operculum. The lack of emergence of larvae from eggs after any of the treatments at 21°C, indicate that temperature is important in stimulating the larvae, whereas the reagents, have a direct physico-chemical effect on the eggshell, possibly destroying the peptide linkages of the protein layers of the egg.

Transmission electron micrographs of eggs at various stages of hatching after incubation in buffer, pepsin/HCl, trypsin and dithiothreitol, all at 37°C were obtained with relative ease due to the increased permeability of the eggs to fixatives (Plates 10-19). The changes observed in the eggshell during hatching in all conditions were similar to the changes found in ageing eggs which spontaneously hatched. This supports the view that the same hatching mechanism is involved but it's rate of occurrence is dependent on the type of stimulation employed. Scanning electron micrographs (Plates 19-20) of eggs indicated that no change occurred to the exterior structure of the shell of hatching eggs except at the operculate region in eggs incubated in pepsin/HCl, trypsin and dithiothreitol. This observation is supported by the video analysis of larval behaviour during incubation in pepsin/HCl, when the larvae suddenly burst out through the operculum in the egg fluid. Photographs of larvae, sectioned and whole (Plates , &), indicate the presence of a membrane around the larvae, which is most probably a sheath, formed from the cast cuticle of the first stage larva, supporting the view that the emergent larva is second stage. Other possibilities include the inner membrane of the egg adhering to

the larva or the slight separation of the outer layer of the cuticle from the body of the larva, perhaps due to an osmotic effect.

The following observations suggested that larvae were actively involved in altering the permeability of the eggshell. Firstly, the increased rate of permeability changes observed in the egg with increasing developmental temperature. Secondly, the accumulation of particulate material between the larva and the lipid layer which may indicate a secretion from the larva, such as an enzyme which is involved in altering the permeability of the eggshell. Thirdly, lack of a staining pattern in ageing non viable eggs in OsO₄, fourthly the ability of larvae to hatch at 21°C, after brief stimulation at 37°C (Section 4.2.3) and fifthly, the increased activity of larvae at 37°C compared to 21°C when observed using a timelapse video system.

A preliminary investigation into the release of proteolytic enzymes by the larvae during hatching was undertaken. Unfortunately the results proved inconclusive, as the increased enzyme activity observed in the egg sample during hatching also occurred to a lesser extent in the control indicating that the proteolytic activity observed may be dependent on the amount of bacterial/fungicidal contamination. Attempts made to sterilize the eggs with various antibiotic /mycotic solutions failed and lack of time precluded further investigations.

From the various experiments performed in this section, the following hypothesis has been formulated, concerning the hatching of *A.tetraptera* eggs. General observations show that changes in the permeability of the egg, in the eggshell wall and the operculum, allow the egg fluid to escape. This reduction in osmotic pressure within the egg causes increased activity of the larva and it's emergence from the egg via the operculum.

It is proposed that exposure of eggs to temperature only, stimulates the larvae alone to alter the permeability of the eggshells. An increase in temperature results in increased stimulation of the larvae, as indicated by the difference in rate of permeability changes and spontaneous hatching in eggs incubated at a range of developmental temperatures. Although not demonstrated in this study, it is suggested that the larvae are stimulated to release enzymes such as lipases and proteases, which alter the permeability of the eggshell layers, allowing the release of egg fluid through the external

uterine layers and causing the collapse of the inner eggshell layers. As the osmotic pressure decreases in the eggs, the eggshell layers are further disrupted by increased activity of the larvae which are no longer inhibited by the osmotic pressure of the surrounding medium, thus resulting in increased dissipation of the egg fluid. The random movements of the larvae and possible proteolytic activity at the modified region of the eggshells where the opercula join to the eggs, weakens the opercula and allows the larvae to emerge from the eggs.

It is suggested that the external factors examined such as pepsin/HCl, trypsin and dithiothreitol, directly affect the physico-chemical structure of the eggshell, by destroying peptide linkages in the egg particularly in the region where the operculum joins the eggshell. This results in rapid permeability changes in the eggs, often to the extent that release of the egg fluid and emergence of the larva both occur through the operculum simultaneously.

The gradual permeability changes which occur in the egg at 21°C, the temperature of development under normal conditions in the animal house, suggest that the larva is preparing for hatching when ingestion by the mouse host occurs. These preparations for rapid emergence may be essential if the larva is to establish itself *in vivo* rapidly, as the egg can pass through the host, within 4 hours (Section 4.2.2). Anya (1966) proposed that the hatching of *A.tetraptera* lay midway between between the freeliving species in which hatching appeared to be internally controlled i.e. the eggs hatched spontaneously once a certain stage of larval development had been reached and, parasitic nematodes which are fully dependent on a stimulus from the host. He postulated that an alteration in the environmental conditions such as a change in pH or temperature, would reactivate the internal clock which causes the mechanism of hatching and thus emergence would result. The experimental results of this study, support the theory postulated by Anya(1966).

CHAPTER 5

DISCUSSION

A reinvestigation of the development and infectivity of *A.tetraptera* eggs was undertaken following problems encountered in obtaining egg hatch using the method described by Anya (1966).

Anya (1966a), using uterine eggs, reported the existence of a period of incubation in the development of infective eggs during which time the larvae though morphologically well developed were incapable of hatching *in vitro* and infecting *in viva*. This delay was not observed in the present study of population egg development with either uterine or faecal eggs. In comparison with Anya (1966a), egg development increased with increasing temperature, although at equivalent temperatures, larval development occurred at a faster rate in both uterine and faecal eggs than found by Anya (1966a). In both studies, an increase was found in the number of embryos which disintegrated with increasing temperature. In addition, in the present study, free larvae were found spontaneously hatched in the incubation medium, their numbers increased with age and temperature. This phenomenon was not reported by Anya (1966a), following his observations on development. Fewer uterine eggs developed in the present study than faecal eggs. This was considered to be due to the immaturity of a proportion of uterine eggs removed from the gravid female, preventing embryonation on incubation.

Uterine and faecal eggs were examined at the individual egg level for any differences in development, permeability and structure which might account for the disparity between Anya's studies (1966a & b) and the present study. No obvious differences were found in egg dimensions, permeability to water, or ultrastructure. Using time lapse video analysis, a delay was observed in the development of uterine eggs compared to faecal eggs. However the time lag of about 6 hours (at 25°C) was insufficient to account for the difference in development between the two studies. Plausible explanations for differences encountered in the two studies include the suggestion that i) the developmental temperatures investigated by Anya (1966a) were inaccurately measured, resulting in a larger margin of error between comparable temperatures than the 1°C reported; or ii) Anya's removal of the "sticky" coat

by washing in 0.01N NaOH, increased the permeability of the uterine eggs, resulting in increased hatchability and iii) different strains of *A.tetraptera* have developed, due to different regimes in individual animal houses.

Uterine *A.tetraptera* eggs are colourless when collected, in comparison to faecal eggs which are brown. This feature is common to many nematode species (Fairbairn, 1957). The brown colouration of the egg, is considered to be due to the tanning of quinnone proteins in the eggshell, possibly as a result of exposure to host gut fluid (Wharton & Barrett, 1979 and Barrett, 1981). It is likely that this process imparts a protective quality to the faecal eggshell. Uterine eggs of the oxyurid, *H.diesingi* (Wharton, 1980) and the ascarid, *A.lumbricoides* (Barrett, 1976) are soluble in acids and alkalis, unlike faecal eggs. Fairbairn (1957) has suggested that the brown colouring of the faecal egg protects the enclosed embryo from sunlight. Ultra-violet light has been shown to have a deleterious effect on the development of the oxyurid eggs of *L.appendiculata* (Dobrovolney & Ackert, 1934) and *E.vermicularis* (Hollaender *et al*, 1940). The same was found to be true of both uterine and faecal eggs of *A.tetraptera*, although the number of faecal eggs which developed was slightly increased. This suggests that some protection may be offered by the faecal eggs under less artificial conditions, when intensity of exposure to ultra-violet light is probably much reduced.

Anya (1966) reported that the major factors which initiated hatching *in vitro* were a pH of 7.3-7.4, using a selection of buffers, a temperature of 37-40°C and an aerobic environment. He obtained maximum egg hatching after 8 hours incubation of eggs. Preliminary experiments on hatching using Anya's method failed to achieve the same rate and level of hatch, instead egg hatching of less than 25% was obtained, after 20 hours incubation. A re-examination of physico-chemical factors which stimulated hatching, found 0.05M zwitterionic "Good" buffers produced optimum levels of hatch (over 80%), although the rate of hatch was still delayed. Explanations for the differences in levels of hatch obtained in the buffers include i) the possibility that the complexing properties of commonly used buffers such as phosphate and borate, may have affected hatching and ii) the concentration of the buffers, inhibited hatching. Eggs incubated in various 0.1M solutions, including Bicine buffer (pH 8.5), experienced a reduced rate of hatch and a decrease in the number of larvae fully emerging. The rate of hatch dramatically increased when eggs were

incubated sequentially in combinations of pepsin-HCl, trypsin and dithiothreitol, indicating that host enzymes may contribute to hatching *in vivo*. Pepsin and trypsin have been found to stimulate infective stages of various nematode species (Lackie, 1975), including several oxyurids (Gordon & Macfie, 1924; Jones & Jacobs, 1941; Hsu, 1951; Chan, 1952 and van der Gulden & van Aspert-van Erp, 1975). The reducing agents, cysteine and sodium dithionite failed to stimulate hatching in *A.tetraptera* eggs unlike dithiothreitol. Ultrastructural observations suggest that its effect on hatching is due to a direct chemical effect on the eggshell, as opposed to altering the redox potential of the egg environment. Cysteine stimulated hatching in the oxyurid *S.muris*, although it was not essential for hatching to occur (van der Gulden & van Aspert-van Erp (1975). CO₂ failed to stimulate hatching *in vitro* in *A.tetraptera* eggs and *S.muris* eggs (van der Gulden & van Aspert-van Erp, 1975). Oxyurid eggs hatch over a wide range of temperature and pH, unlike ascarid eggs which also require CO₂ for hatching *in vitro* (Rogers, 1962). The different requirements for hatching of oxyurid and ascarid eggs *in vitro* may reflect a requirement for differing host stimuli *in vivo*.

An investigation of *in vivo* hatching in the present study, found partially emerged and fully emerged larvae in the stomach and along the length of the small intestine 15 minutes post infection and in the caecum 30 to 60 minutes post infection. Philpot (1924) and Anya (1966b) recovered larvae from the posterior end of the small intestine and the caecum, 2 to 4 hours post infection. Although Anya (1966b), recovered no larvae from the stomach or small intestine, he indicated that the initiation of hatching must occur at an earlier stage, as suggested by the results of this study. The difference in rate of hatch observed in the studies may reflect differences in the age of the infected mice used. The observation that *A.tetraptera* hatching commenced in the stomach and upper intestine may suggest the involvement of HCl and the enzymes, trypsin and pepsin *in vivo*, reflecting the results found *in vitro*. The oxyurid *E.vermicularis* contained in capsules also hatched in the stomach (Cobb, 1890; cited Schwartz, 1923). Silverman and Podger (1964) and Parker and Croll (1976) suggested host enzymes were essential for exsheathing *in vitro*. The difference in rate of hatching *in vivo* and optimum hatching *in vitro*, suggests that other conditions present in the mouse gut are important for stimulating hatching. The permeability changes observed in ageing egg, may aid the rapid eclosion of larvae *in vivo*, increasing the chances of

establishment of infection.

Spontaneous hatching of *A.tetraptera* eggs has been reported in studies by Anya (1966) and Hsu (1951), but was not investigated. Anya (1966) reported the occurrence of background hatching (2-8%) in unstimulated control eggs (aged 9-10 days), at 25°C. This level of hatch, corresponds to that obtained for spontaneous hatching in the present study in similarly aged eggs at 25°C. Hsu (1951) reported that the majority of larvae had hatched from *A.tetraptera* eggs incubated in water at 28°C, after a 3 month period. He also found *E.vermicularis* eggs hatched after 3 days incubation in water at 28°C. Background or spontaneous hatching has also been observed in *A.lumbricoides* (Rogers, 1960 and Fairbairn, 1961) and *G.rostochiensis* (Ellenby and Perry, 1976), nematodes which normally require stimulation from the host to hatch.

Gradual permeability changes were detected in ageing *A.tetraptera* eggs, by i) the development of larval shrinking in ageing eggs incubated in concentrated NaCl solutions and, the decreased levels of hatch obtained with age when stimulated to hatch; ii) differential staining of ageing eggs by OsO₄ and iii) ultrastructural studies. Several possibilities have been discussed in Section 4.3.3., concerning the interpretation of the results. The following hypothesis was considered most probable. Initially, the structure of the internal uterine layer is altered, possibly by the breakdown of lipo- proteins and the underlying layers separate from this layer or the vitelline layer. Secondly a change in permeability appears to occur in the lipid layer, allowing release of the eggfluid, resulting in collapse of the chitin and lipid layer and the possible uptake of water by the larva. A change in the permeability of the chitin layer probably occurs at some stage as suggested by the deformation which occurs to its structure during hatching. A further change in permeability occurs when the operculum opens allowing larval emergence.

The increased rate of permeability changes in ageing eggs with increased development temperature indicates that temperature is involved in the gradual change in permeability of the eggshell prior to stimulation to hatch. The increased number of free larvae found in the incubation medium with increased temperature, indicates that temperature is important in stimulating the larvae to emerge. The increased rate of hatching observed in ageing eggs once stimulated by incubation in buffer at 37°C, suggests that the changes demonstrated in the egg with age represent the initial stages of the hatching

process. This early change in permeability which occurs in the egg once the larva has embryonated has also been reported in *Ancylostoma* species, once the larvae become active within the egg (Matthews, 1985). Hurley & Sommerville (1982) reported that *A.lumbricoides* eggs aged 20 days old stained in iodine, whereas 0 day old eggs were impermeable to iodine (Barrett, 1976). They acknowledged the possibility that small amounts of hatching may be produced by embryonated eggs, leading to gradual changes in permeability, but considered it unlikely.

Changes of permeability investigated in other nematodes have been reported to occur shortly before hatching commenced in "spontaneous" hatching nematodes or after stimulation to hatch in "infective" nematodes (Clarke & Perry, 1981). Anya (1966) reported a change in permeability of the eggshell, shortly before hatching commenced, after observing increased plasmolysis of eggs in salt solutions following stimulation to hatch. In the oxyurid, *S.muris*, van der Gulden and van Aspert-van Erp (1976) demonstrated an increase in eggshell permeability in the initial stages of hatching.

The same permeability changes found in ageing eggs were observed in eggs stimulated to hatch in various treatments such as buffer, pepsin-HCl, trypsin and dithiothreitol, all at 37°C, but their rate of occurrence was increased. Larval shrinking within the egg in NaCl solution, staining in OsO₄ and ultrastructural studies provided the following evidence. At 37°C, permeability of the eggshell increased most rapidly following incubation in pepsin-HCl, then trypsin and dithiothreitol and lastly buffer. Pepsin and trypsin, failed to alter the permeability of eggs at 21°C, indicating that these enzymes function more efficiently at body host temperature. Bicine buffer (pH 8.5), had no effect on the permeability of the eggshell at 21°C, indicating that temperature was the cause of the permeability changes when eggs were incubated in buffer at 37°C. The failure of larvae to emerge at 21°C, suggests that temperature is important in stimulating the larvae to leave the egg. Dithiothreitol and HCl, both increased the permeability of the eggshell at 21°C and the observation that larval staining occurred in the region nearest the operculum, suggested that they directly affected the permeability of the operculum. Scanning electron micrographs indicate that no change occurred to the surface of eggs incubated in pepsin-HCl, trypsin and dithiothreitol except at the opercular region. As the exterior uterine layer retains its

structure during hatching, it indicates that the increased permeability of the egg occurs as a result of changes to the underlying layers. The rate and pattern of staining in OsO_4 and the rapidity of breakdown of the internal uterine layer as indicated by the transmission electron micrographs, suggest that the various stimuli are able to enter the egg via the pores in the external uterine layer. The physico-chemical reagents probably have a direct effect on the peptide linkages of proteins in the eggshell. Van der Gulden & van Aspert-van Erp (1976) observed from scanning electron micrographs of hatched *S.muris* eggs that trypsin affected the operculum of the eggshell.

Several observations suggest that larvae may be actively involved in the permeability changes of the eggshell. These include i) the increased rate of permeability change within the egg at increased temperature, ii) the abnormal staining pattern in non viable eggs, iii) the increased softening of the eggshell in ageing eggs, iv) the increased activity of the larva with temperature, v) the accumulation of particulate material between the lipid layer and the larva and vi) the ability of the larva to hatch at 21°C, after brief stimulation at 37°C. An assay for proteolytic enzymes proved inconclusive due to bacterial and fungal contamination. Enzyme involvement has been suggested in the hatching of a number of other nematode species (Taylor, 1962; Flegg, 1968; Bird, 1968; Bridge, 1974; Croll, 1974). Direct evidence for the involvement of enzymes in hatching has been demonstrated in *A.lumbricoides* (Rogers, 1958 & 1960; Fairbairn, 1961; Ward & Fairbairn, 1972 and Hinck & Ivey, 1976), *H.contortus* (Rogers & Brooks, 1977) and *H.glycines* (Tefft & Bone, 1985).

Perry (1987), has suggested that eggshell digesting enzymes need not necessarily originate from the juvenile, but may be located in the egg fluid or the eggshell, awaiting activation. The presence of enzymes in the secretion filling the spaces of the internal uterine layer may account for the observation that the initial alteration to the eggshell occurs in the internal uterine layer, although the lipid layer is probably intact, preventing enzymes in the eggfluid or secretions by the larva from reaching the outer layers of the eggshell. If this is the case, the enzymes originate from the gravid female, as the uterine layers are secreted by the female during the passage of the egg through the uterus (Wharton, 1979). Studies on the affect of photoperiod on diapausing (Evans, 1982 and Hominick *et al*, 1986a & b) and hatching eggs (Bird *et al*, 1980) have demonstrated the involvement of the female nematode. The

possibility arises that the changes to the egg layers of *A.tetraptera* emanate from outside the egg. Perry & Trett (1986), failed to detect the lipid layer in eggs from cysts of *H.schachtii* contaminated with fungi. It is possible that fungal enzymes enter the egg and disrupt the lipid layer. Bacteria and fungi are often found in close proximity to the sticky outer coat of *A.tetraptera* eggs in unsterile solutions and during passage through the host hind gut and, may, affect the permeability of the eggs.

Another possibility, is that a structural alteration of the layers occurs. Clarke and Perry (1985a & b), have recently indicated that hatching agents may alter membrane permeability by replacing or binding Ca^{2+} in lipoprotein membranes of the eggshell of *G.rostochiensis* and *H.schachtii* eggs. Thus the hatching mechanism of *A.tetraptera* eggs may be Ca^{2+} dependent.

The ultrastructural observation that the lipid layer apparently remains intact during hatching may suggest that changes in permeability of this layer are due to larval movement. This view has been postulated for various nematode species including *T.retortaeformis* (Wilson, 1958), *M.javanica* (Wallace, 1966 & 1968) and *A.lumbricoides* (Barrett, 1976). Matthews (1986) found *Ancylostoma* larvae were active for several hours before emergence. He considered a mechanism which involved cumulative activity to emulsify the lipid layer without the participation of some enzymatic activity unlikely.

Increasing osmotic concentration was found to reduce the rate of hatch and number of larvae which emerged from *A.tetraptera* eggs. A similar effect, including reversible inhibition, has been observed in other nematode species (Wallace, 1955 & 1966; Dropkin *et al*, 1958; Wilson, 1958; Croll, 1974; Reversat, 1975; Clarke & Perry, 1980). The initial embryonation of *A.tetraptera* eggs was apparently unaffected by osmotic stress, but the eggs failed to hatch when incubated in buffer solution at 37°C. Larval shrinkage which occurred in ageing eggs incubated in concentrated salt solutions was also irreversible. This effect was not reversed when eggs were incubated for hatching in 0.05M Bicine buffer solution. Matthews (1985) also found osmotic stress directly affected the behaviour and development of *Ancylostoma* larvae after the active stage was obtained. Croll (1974) however, found the development of motile *N.americanus* larvae was unaffected by osmotic stress. The osmotic effect observed in *A.tetraptera* egg hatching, suggests that water permeability may

be important in the hatching mechanism.

Video analysis of larval behaviour indicated that the larvae within the egg swelled prior to hatching, suggesting an influx of water into the egg. Indirect evidence for water movement into the egg and/or juvenile prior to hatching have been found in other nematode species (Wilson, 1958 and Croll, 1974) including the oxyurid *S.muris* where van der Gulden & van Aspert-van Erp (1976) considered a change in permeability of the egg resulting in water uptake by the larva to be essential for the operculum to open. Direct evidence of larval hydration after stimulation and prior to eclosion has been found in *G.rostochiensis* (Ellenby & Perry, 1976), *H.goettingiana* (Perry *et al*, 1983) and *Ascaris suum* (Clarke & Perry, 1980). Various mechanisms have been postulated concerning the function of water uptake in hatching, including 1) an increase in hydrostatic pressure within the egg, due to water uptake by the egg contents causes the eggshell to rupture (Looss, 1911; Wilson, 1958 and Croll, 1974 & 1975); 2) loss of solute (trehalose) from the egg, removes osmotic stress on the unhatched larva, allowing uptake of water by the larva, the initiation of larval activity and subsequent emergence (Clarke *et al*, 1978; Clarke & Perry, 1980 and Perry *et al*, 1983) and 3) an increase in eggsize allows the uptake of water into the egg, which dilutes the contents, reducing the osmotic stress on the larva and allowing larval motility and hatching (Banyer & Fisher, 1980).

The involvement of water uptake in the hatching of *A.tetraptera* eggs is uncertain. The disappearance of egg fluid stain from the egg, swelling of the larva and active involvement of the larva when escaping suggest that the second mechanism may apply. However larvae have been observed to burst from eggs incubated in pepsin-HCl, suggesting the rapid change in permeability of the egg, resulted in a rapid influx of water into the egg which increased the internal hydrostatic pressure rupturing the operculum.

Anyia (1966) suggested that the hatching of *A.tetraptera* was comparable to plant parasitic and free living nematodes rather than ascarids, because of it's ability to hatch in a wide range of stimuli. He postulated that *A.tetraptera* egg hatching lay midway between free living species, when eggs hatch spontaneously once a certain stage of development is reached and parasitic species which rely upon a host stimulus for hatching. The results of the

present study which demonstrate a gradual change in the permeability of ageing eggs appears to support this suggestion. It would be interesting to investigate whether gradual permeability changes occur in other oxyurids and, "infective" nematode species which also show spontaneous hatching in a proportion of the egg population.

Temperature has been shown to be important in the permeability changes which occur within the egg, both gradual and rapid. It is not known whether it directly affects the membrane lipids of the eggshell (Thompson and Huang, 1980) or activates a receptor in the larvae, such as heat shock genes (van der Ploag *et al*, 1985) which cause a neurosecretory response via the endocrine system. Davey & Goh (1984) and Davey (1984) have provided evidence that the secretion of exsheathing fluids in *Phocanema decipiens* is under the control of the endocrine system. Sommerville and Rogers (1987) have postulated that the endocrine system is involved in the hatching of nematode eggs. The host stimulus acts as a physiological trigger, activating a receptor in the larva probably through an intermediary system which affects target cells, leading to the secretion of hatching enzymes. Neurosecretory cells and neurosecretory granules have been found in *A.lumbricoides* larvae, in the vicinity of the nerve ring, but their function is unknown (Davey, 1964 and Rogers, 1968).

The possibility that other members of the *Oxyuroidea*, share the same ability as *A.tetraptera* eggs to alter their permeability over a wide range of temperatures, would account for the success of the family to parasitize both poikilothermic and homoiothermic hosts.

CHAPTER 6

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Plate 1

A. Faecal eggs incubated at 25°C for 24 hours containing developed larvae.
Mag.x960. SB.=25um.

B. Faecal eggs containing undeveloped embryos prior to incubation at 25°C. Mag.x960. SB.=25um.

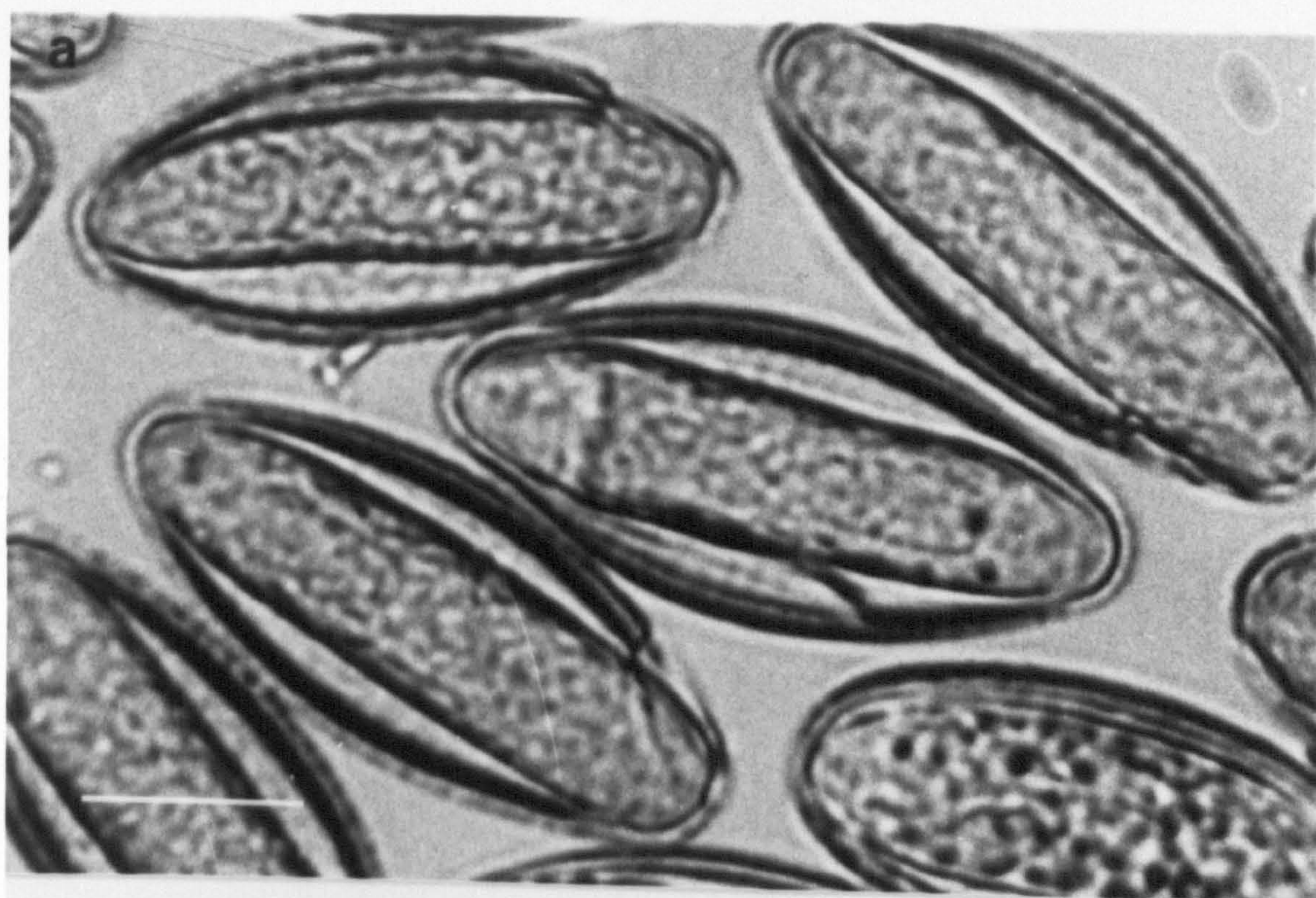


Plate 2

Larva emerging from an egg incubated in 0.05M NaCl solution at 37°C after 5 days development at 25°C in 0.05M NaCl solution. The thin membrane surrounding the larva, may either represent a moulted cuticle or the inner layer of the egg. Mag.x1200. SB.=20um.

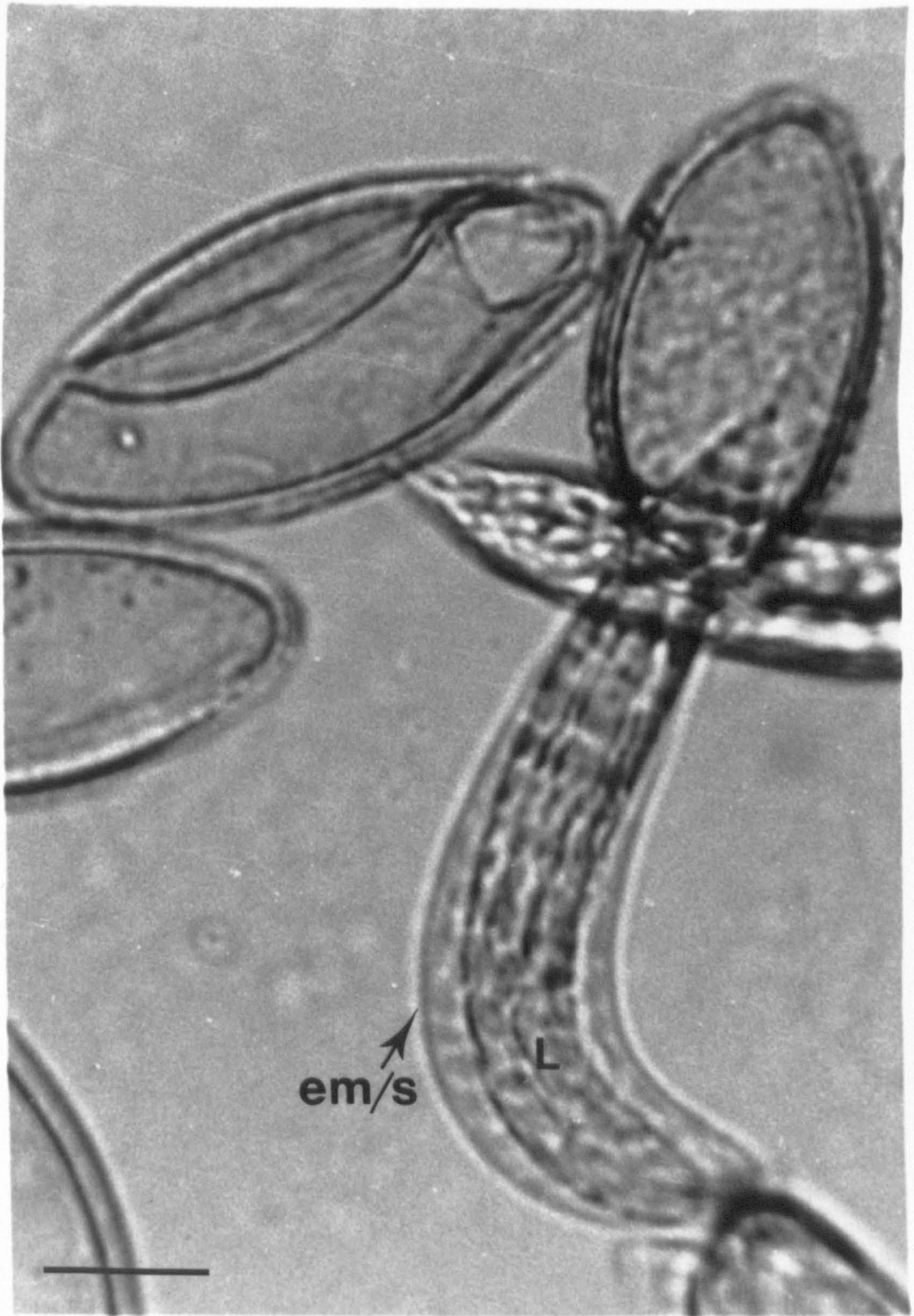


Plate 3

A.Thin section through a fully formed faecal eggshell. The shell consists of the external uterine layer (el), internal uterine layer(il), vitelline layer (vl), chitinous layer (cl) and the lipid Layer(l). Nomenclature according to Wharton (1979). Mag.x19000.SB.=1um.

B.Section through eggshell wall at increased magnification.Note the struts and spaces of the internal uterine layer (il). Mag.x48000. SB.=500nm.

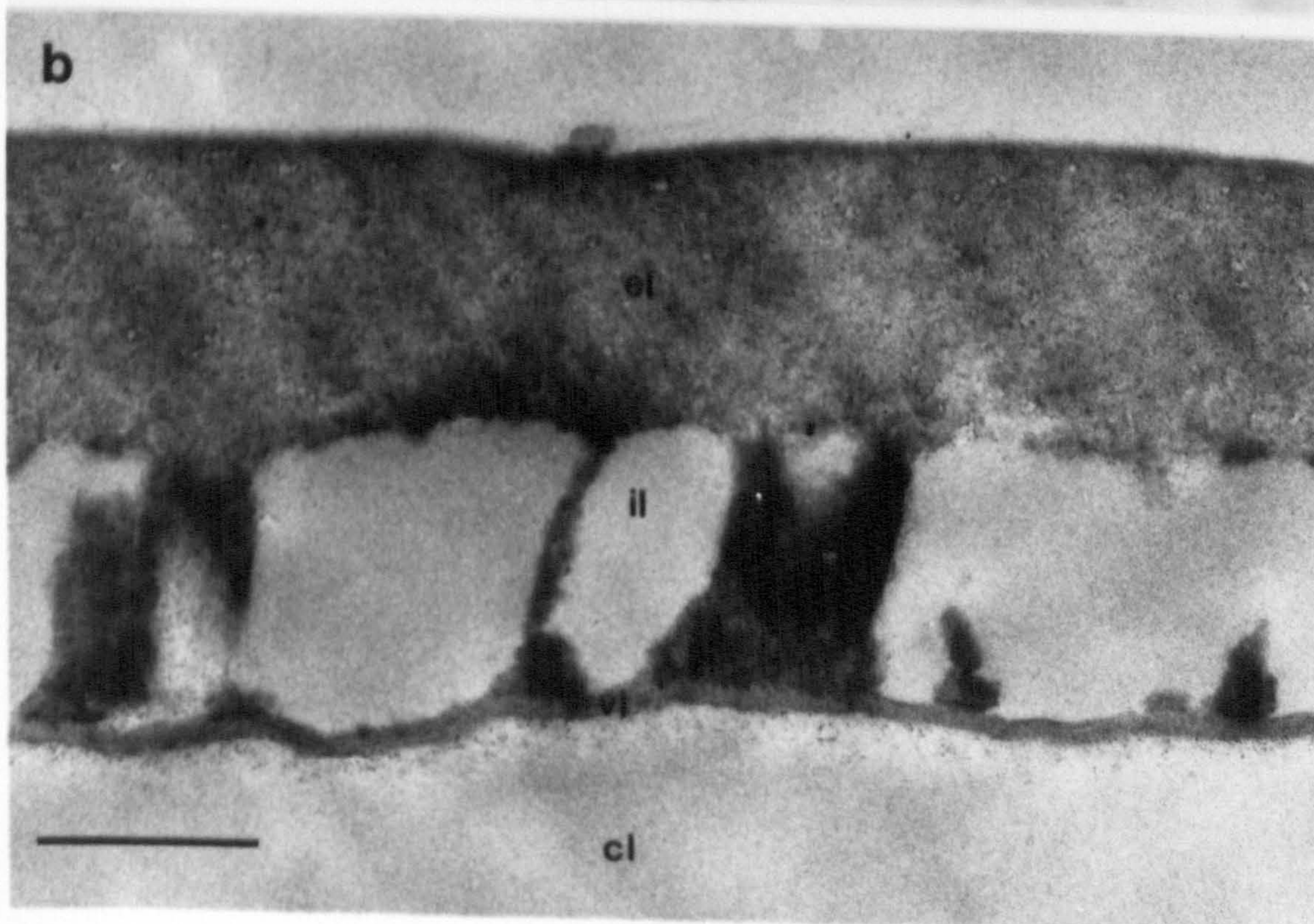
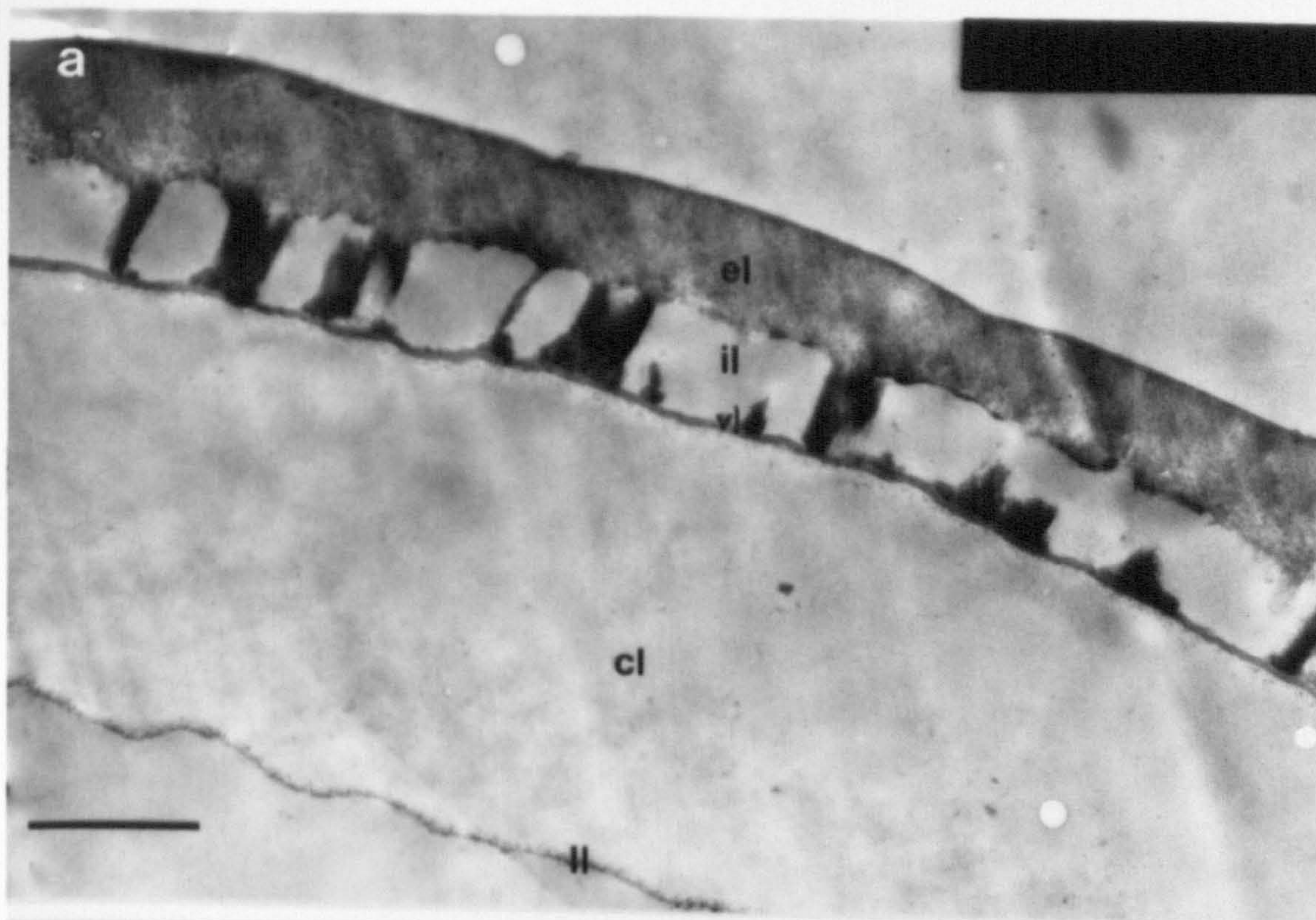


Plate 4

A.SEM of cryofixed faecal egg. Note the evenly sculptured shell and operculum (op). Mag.x1500 SB.=20um.

B.SEM of cryofixed uterine egg. In comparison to the faecal egg, note the smooth appearance of the shell (arrow) and particulate material (pm) sticking to the outer layer. Mag.x1725. SB.=20um.

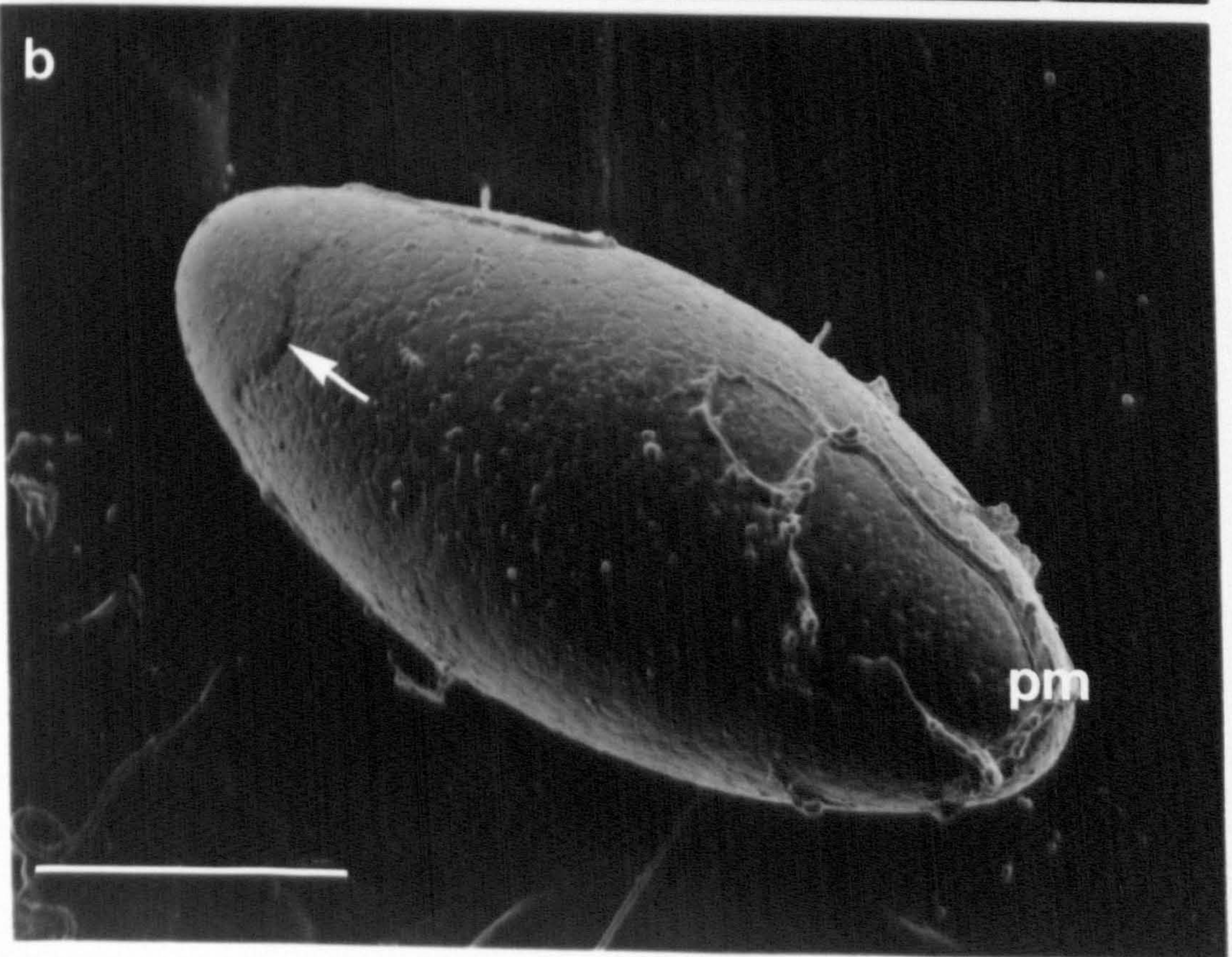
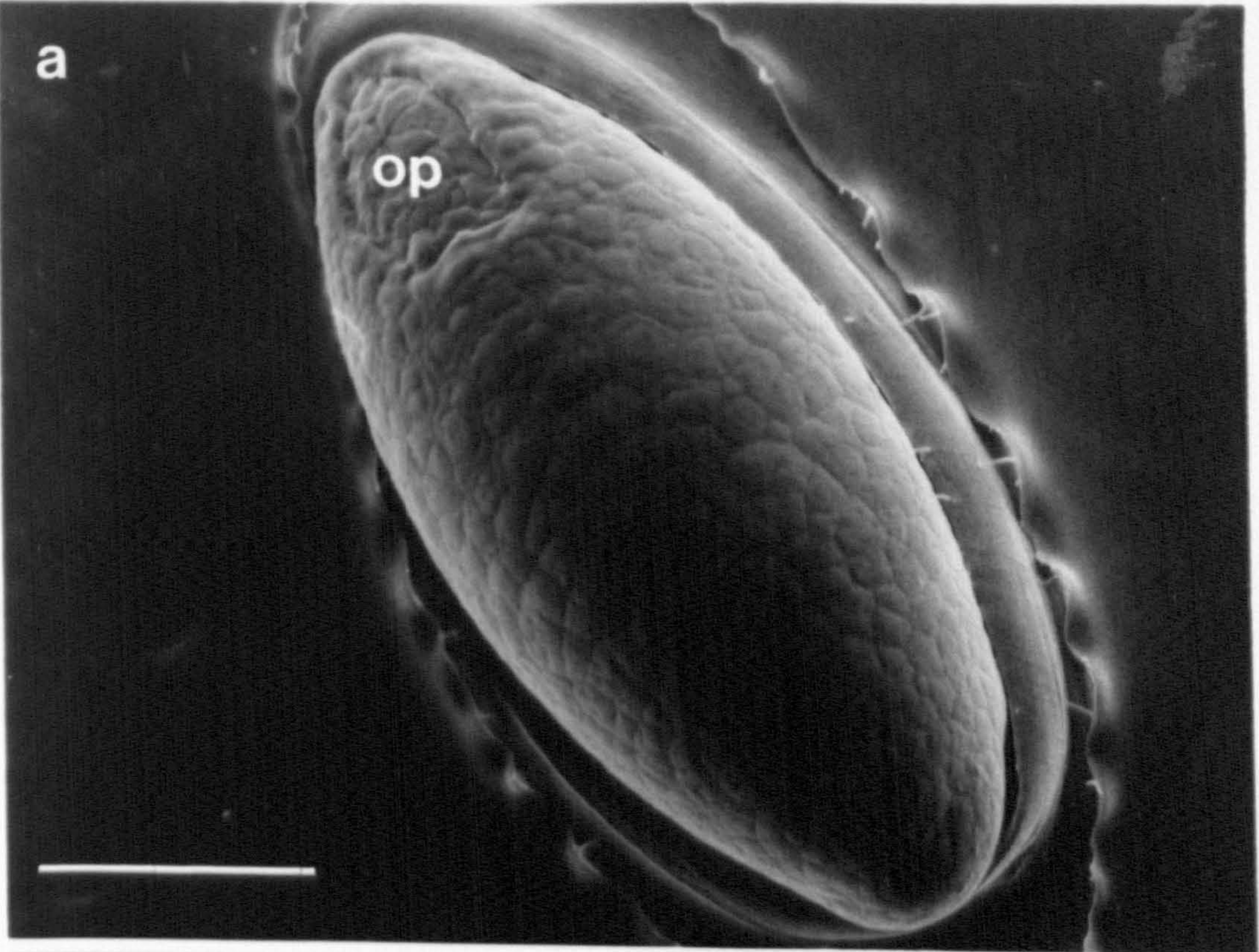


Plate 5

A.Thin section through an unembryonated egg. Mag.x42500. SB=400nm.

B.Section through opercular (op) region of the unembryonated egg (Plate 5A). Note the narrowing of the external (el) and internal (il) uterine layers. Mag. x13500, SB.=1um.

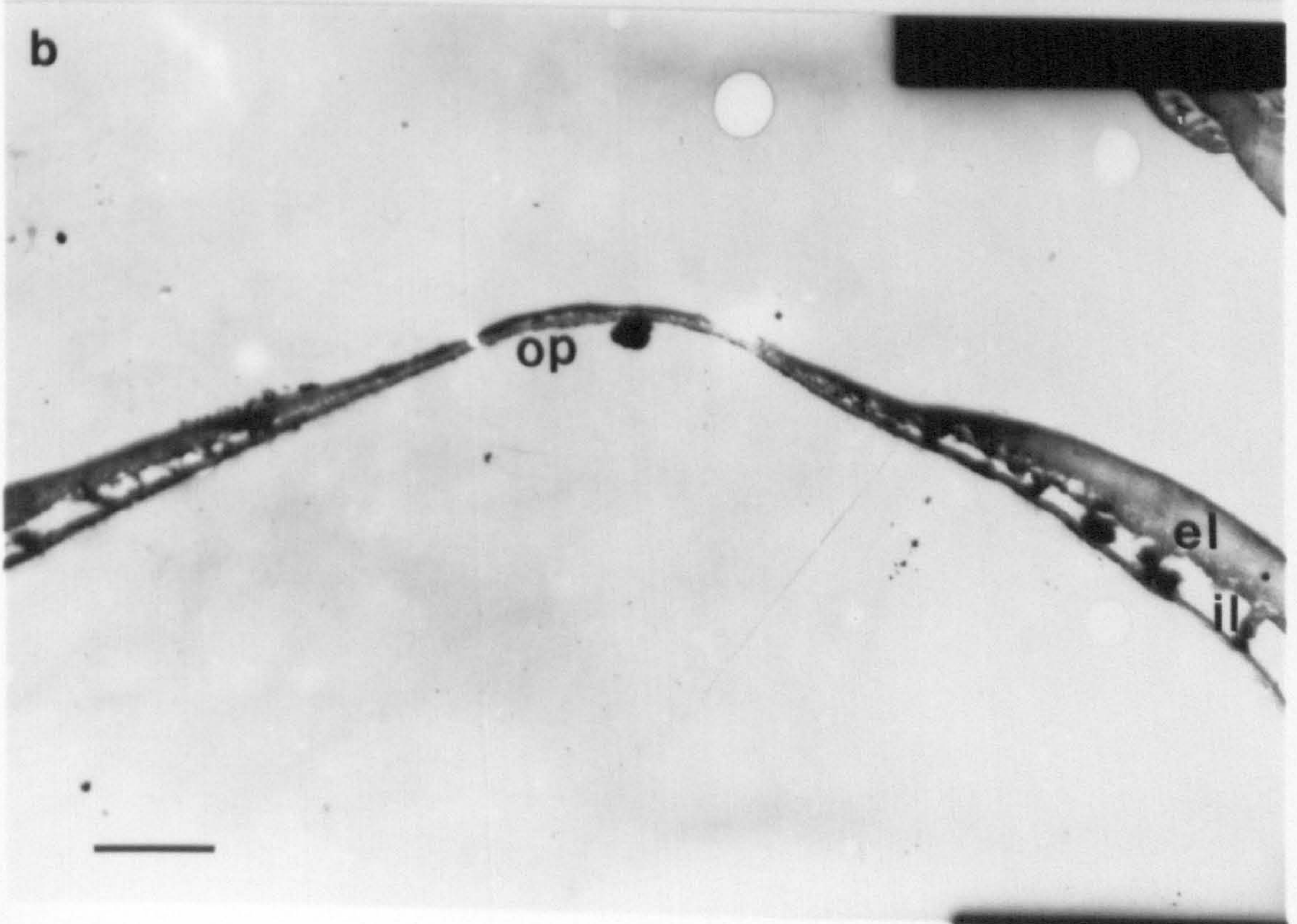
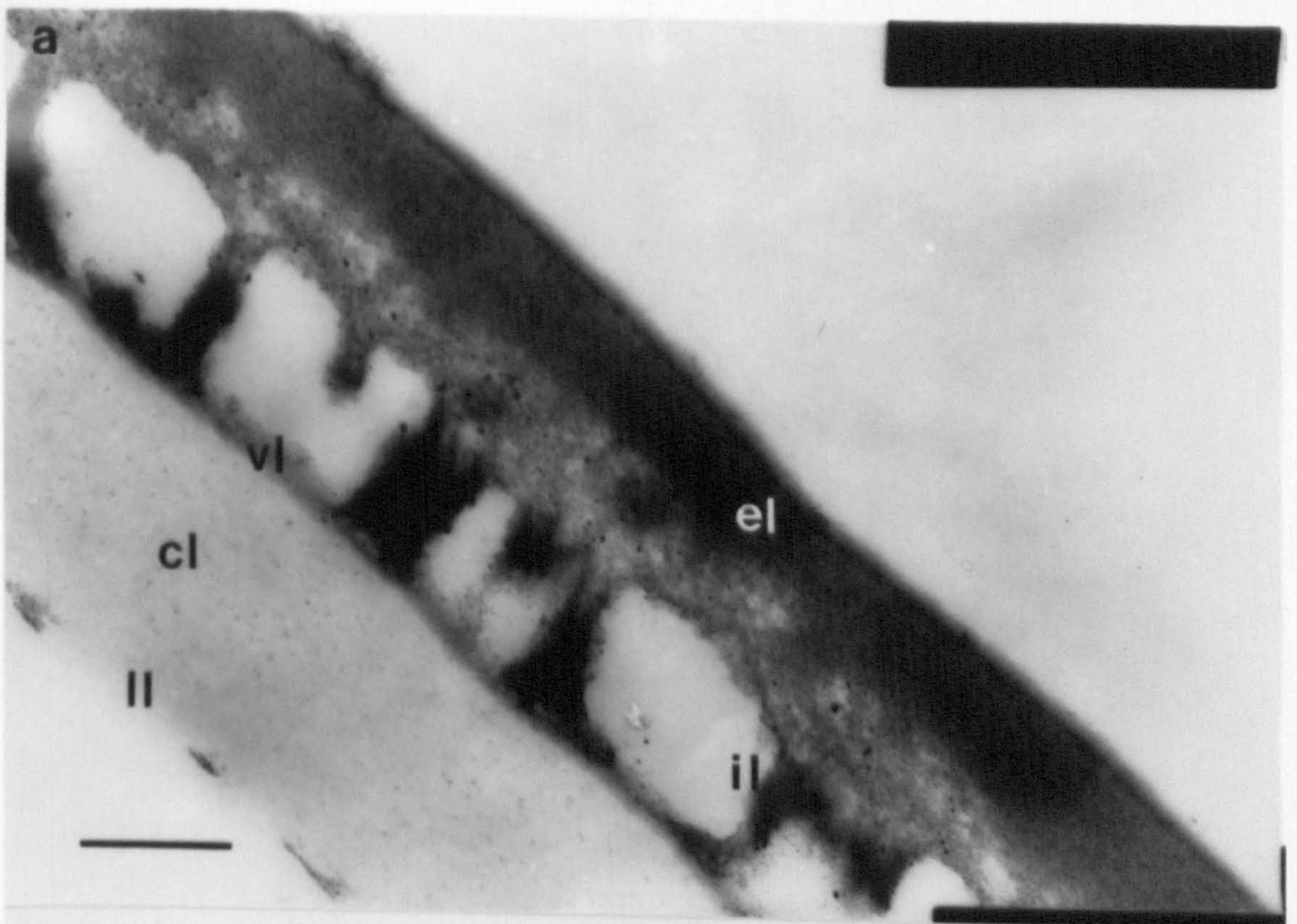


Plate 6

A.Thin section through whole egg, previously incubated at 21°C for 5 days. Note the intact operculum (op) and space (sp) where the chitin (cl) and lipid (ll) layer have separated from the above disintegrating internal uterine layer (il). Mag.x3000. SB.=6um.

B.Increased magnification of the intact operculum of the egg in Plate 6A. Mag.x5200. SB.=5um.

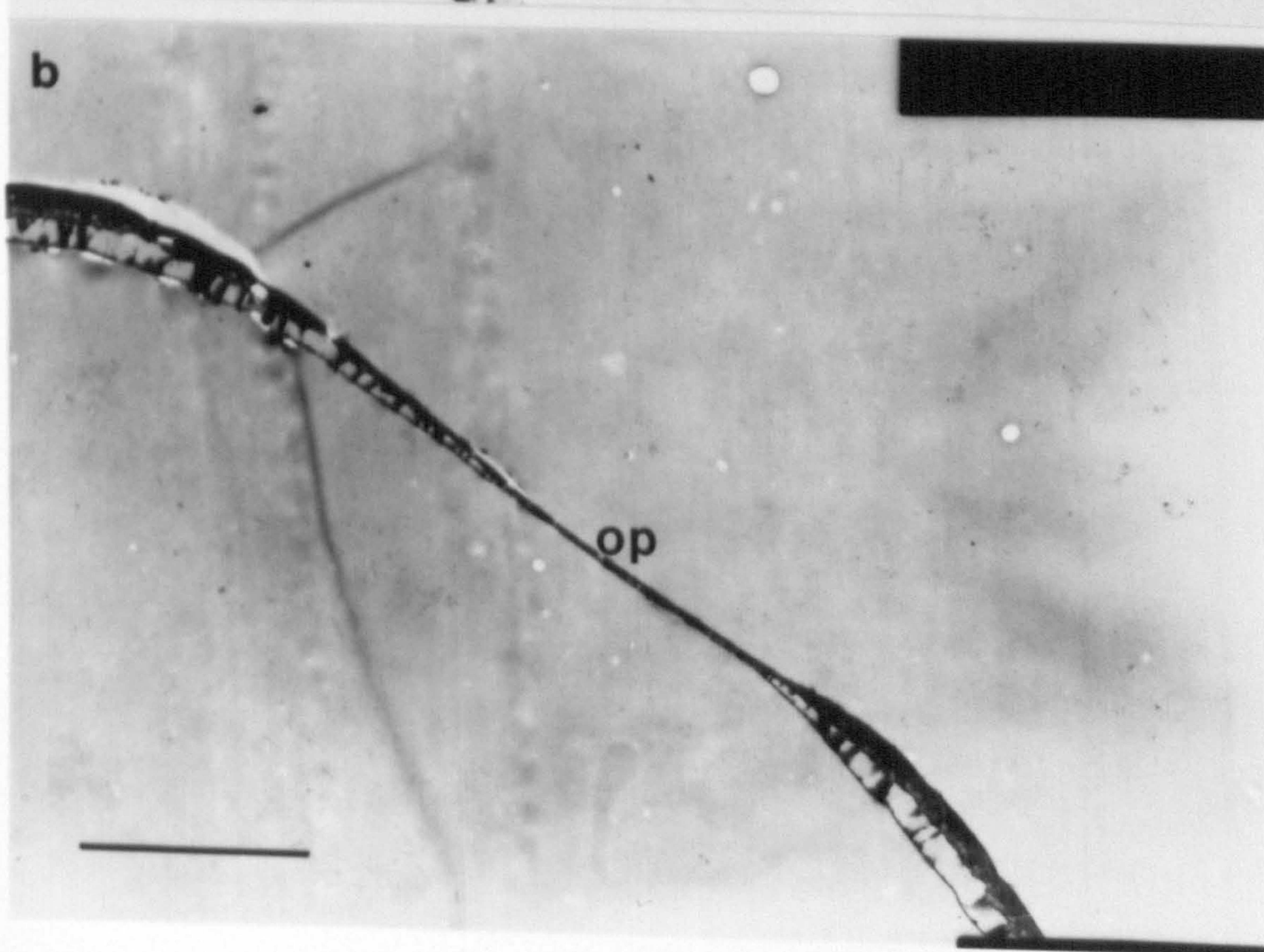
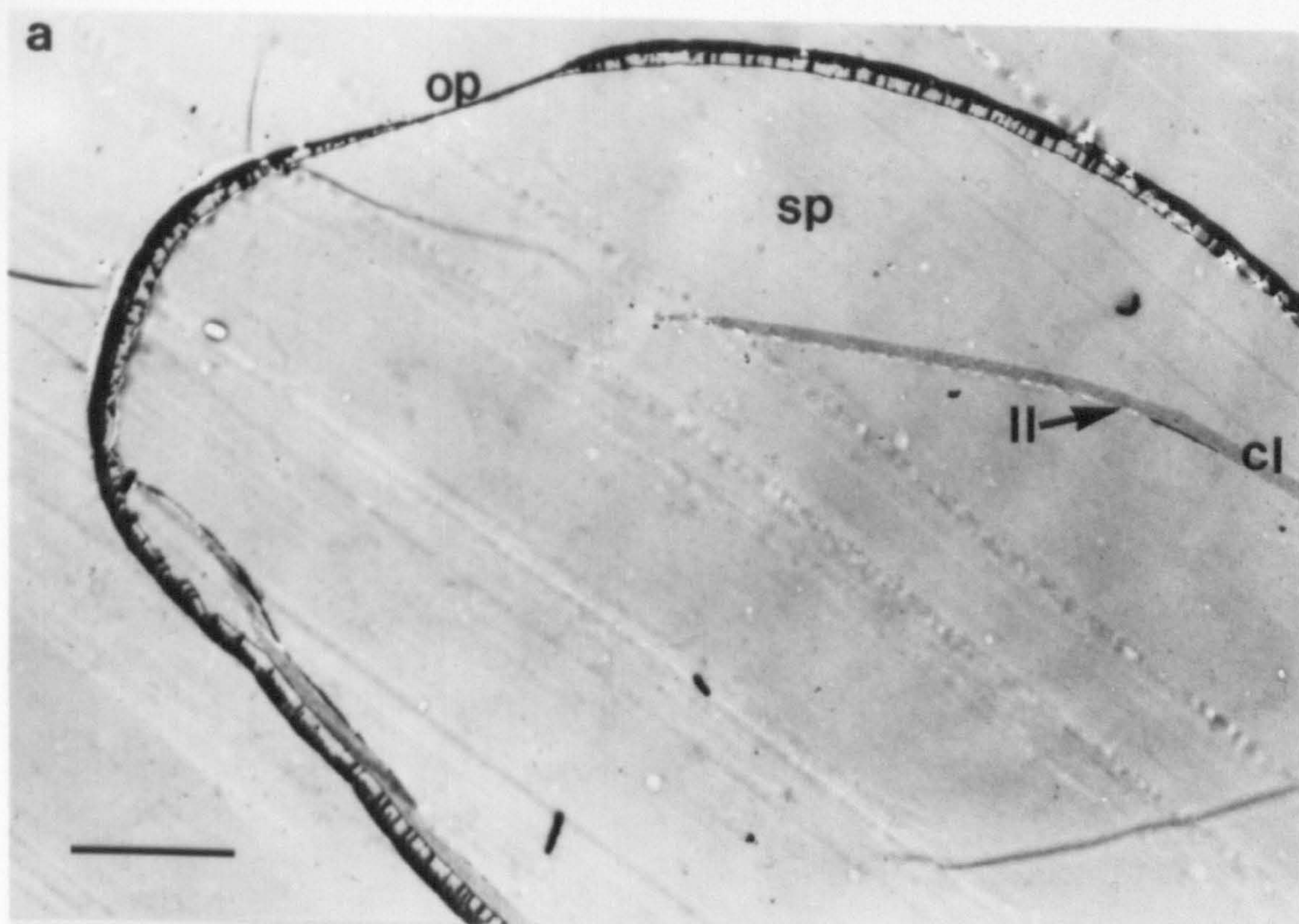


Plate 7

A.Thin section through an egg aged 5days old in which the vitelline layer (vl) has separated from the disrupted internal uterine layer (il). Mag.x12500. SB.=1um.

B.Thin section through an egg aged 7 days old. Note the disintegration of the chitin layer (cl) and separation of the underlying lipid layer (ll) forming a space (sp). The operculum (op) has collapsed inwards and the internal uterine layer (il) has disintegrated in the vicinity of the operculum. Mag.x5000. SB.=5um.

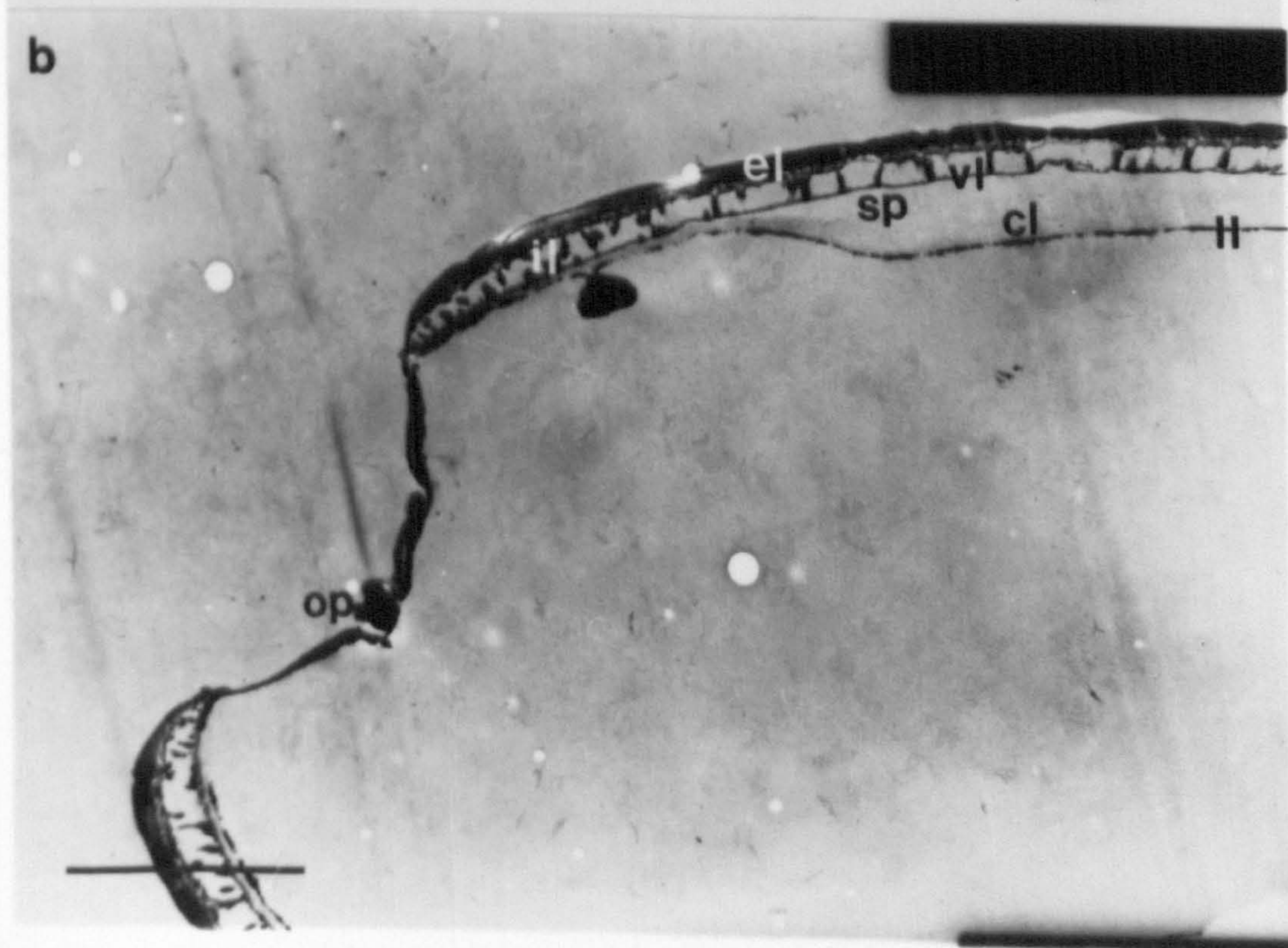
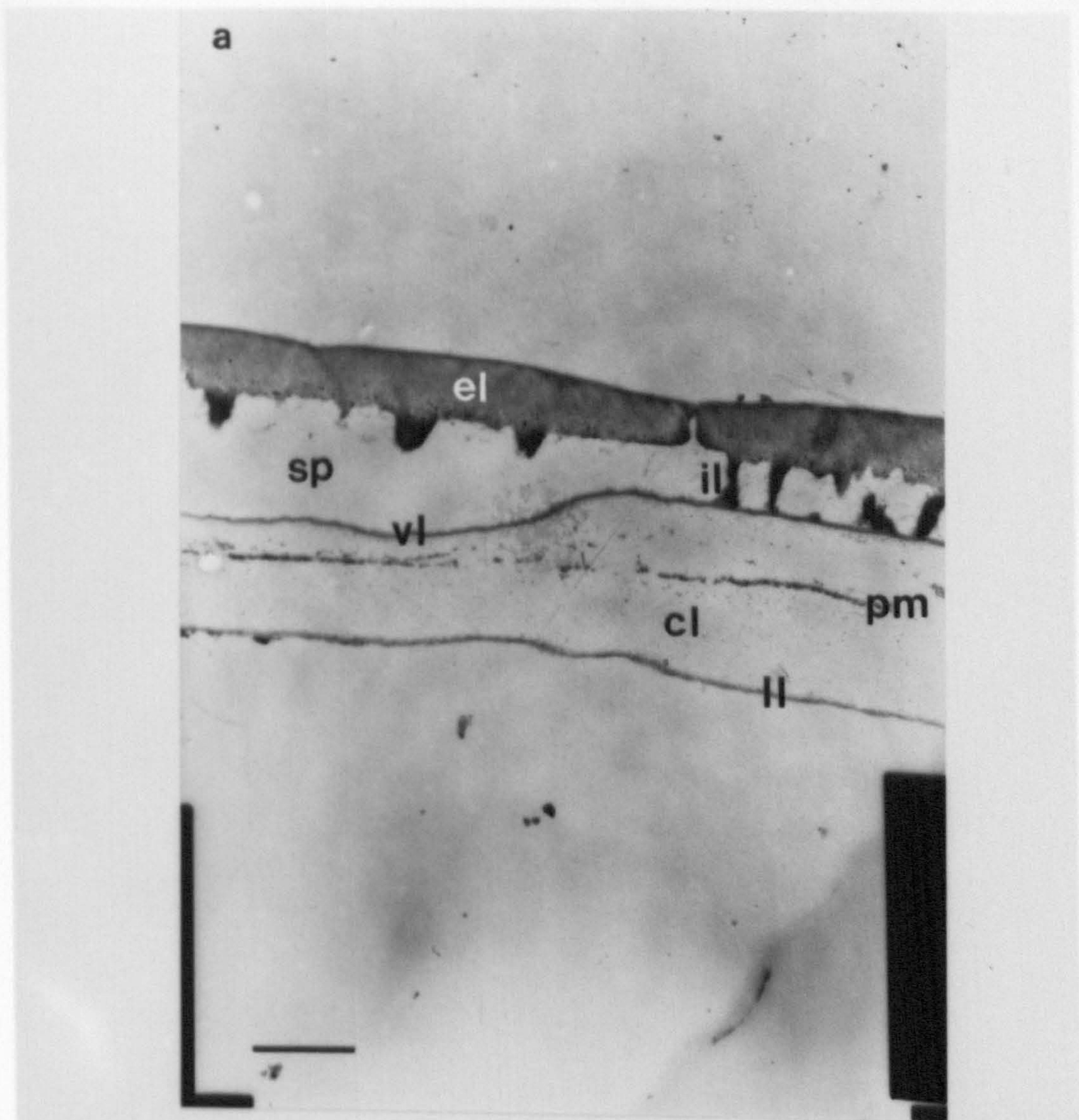


Plate 8

A.Increased magnification of eggwall of egg (Plate 7b). Note the blackened osmium deposits along the lipid layer (ll) and the chitin layer (cl) separating from the vitelline layer (vl). Mag.x32000. SB.500nm.

B.Enclosed larva of a 10 day old egg has stained black due to contact with OsO_4 stain. The struts of the internal uterine layer (il) have disintegrated, the chitin layer (cl) has separated from the vitelline layer (vl) and the lipid layer (ll) has either collapsed inwards lying in close proximity to the larva or it has disintegrated. The operculum (op) has partially disintegrated. Mag.x3200. SB.=5um.

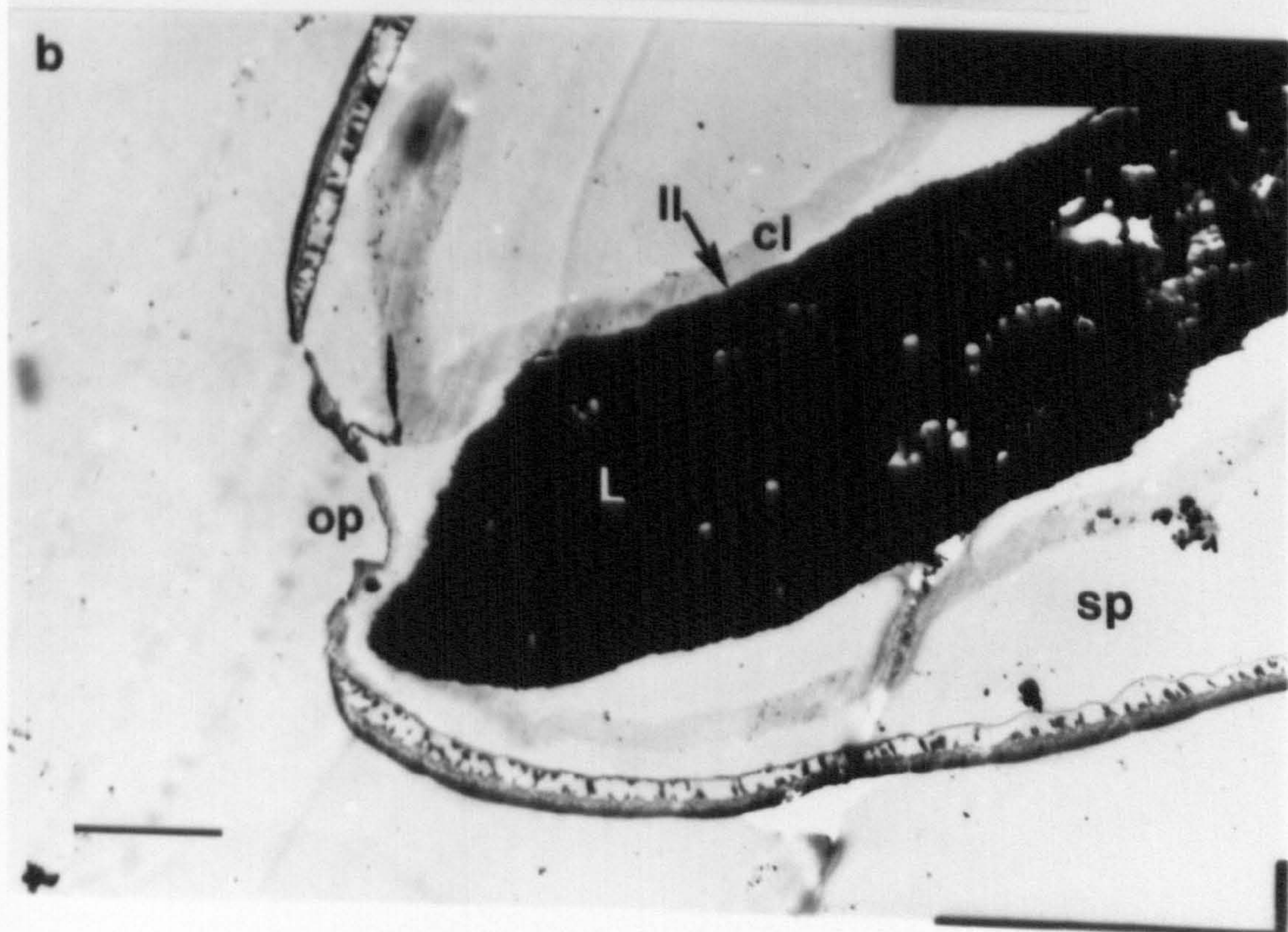
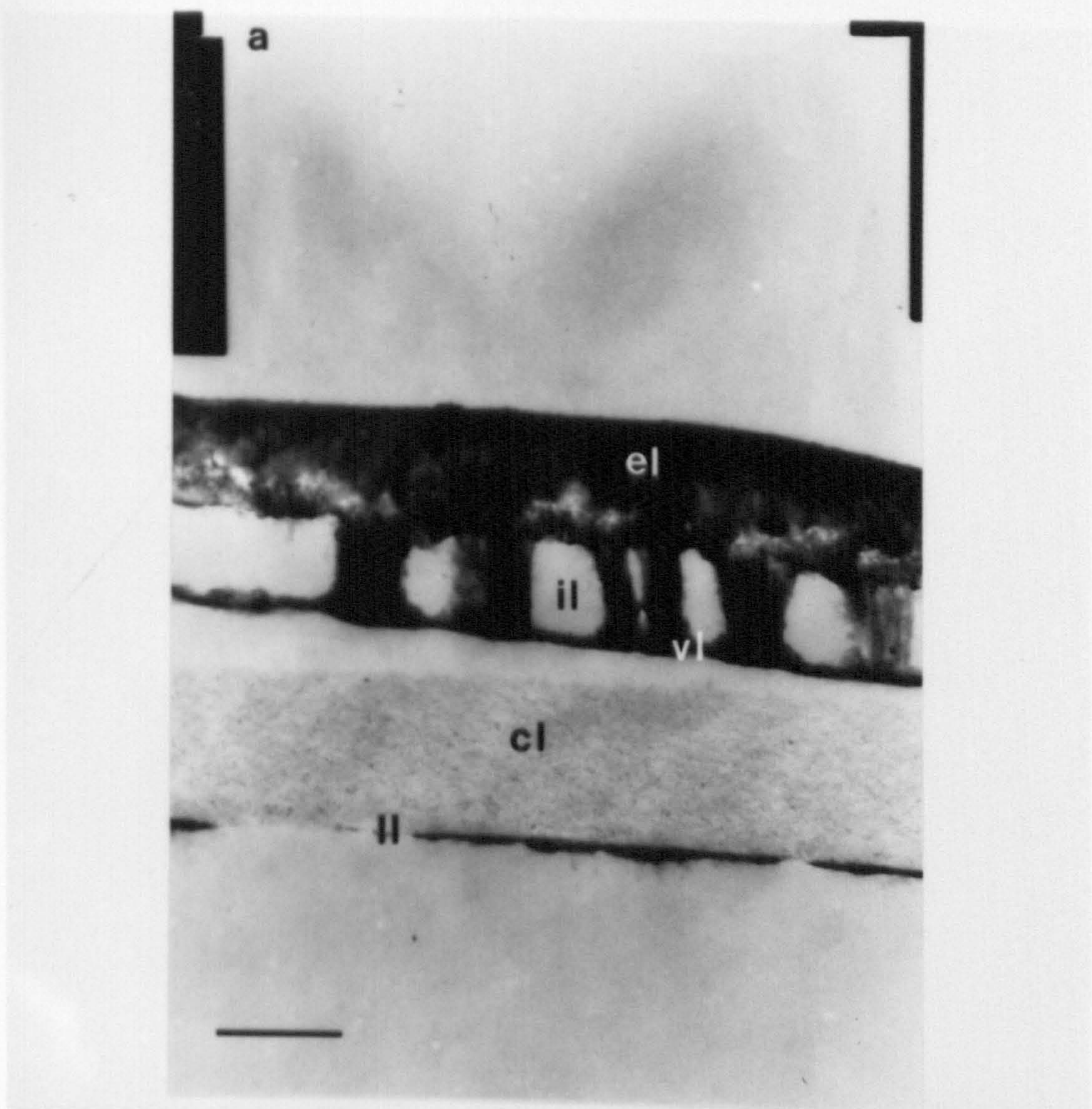


Plate 9

A. High powered magnification of the disintegrating operculum (op) of the egg in Plate 8B. Note the electron dense portion below the operculum. Mag.x17500. SB.=1um.

B. Increased magnification of the egg in Plate 9, showing the collapsed chitin layer (cl), portion of attached lipid layer (ll) and the breakdown of the operculum (op) and internal uterine layer (ll). Mag.x10000. SB.=2um.

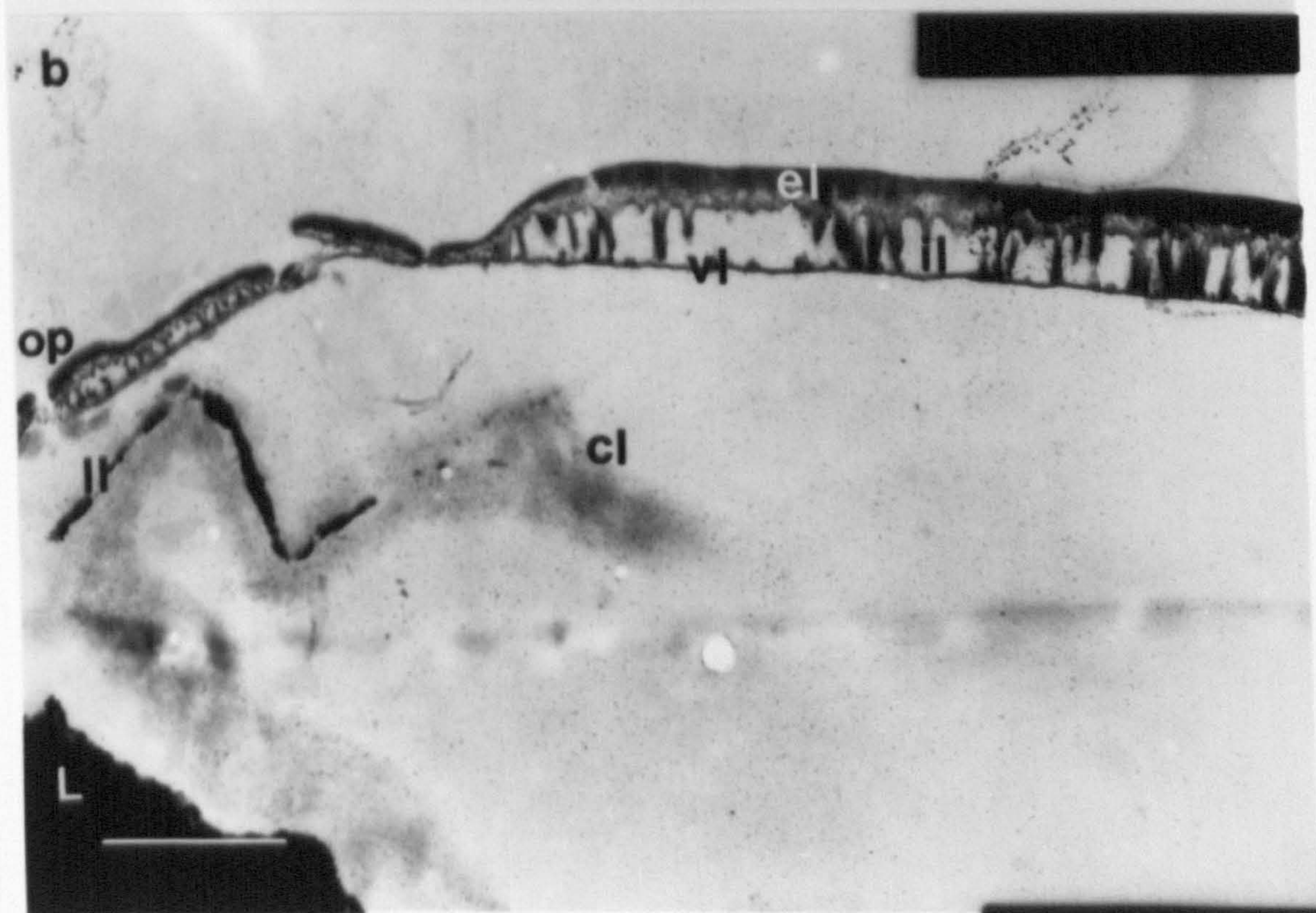
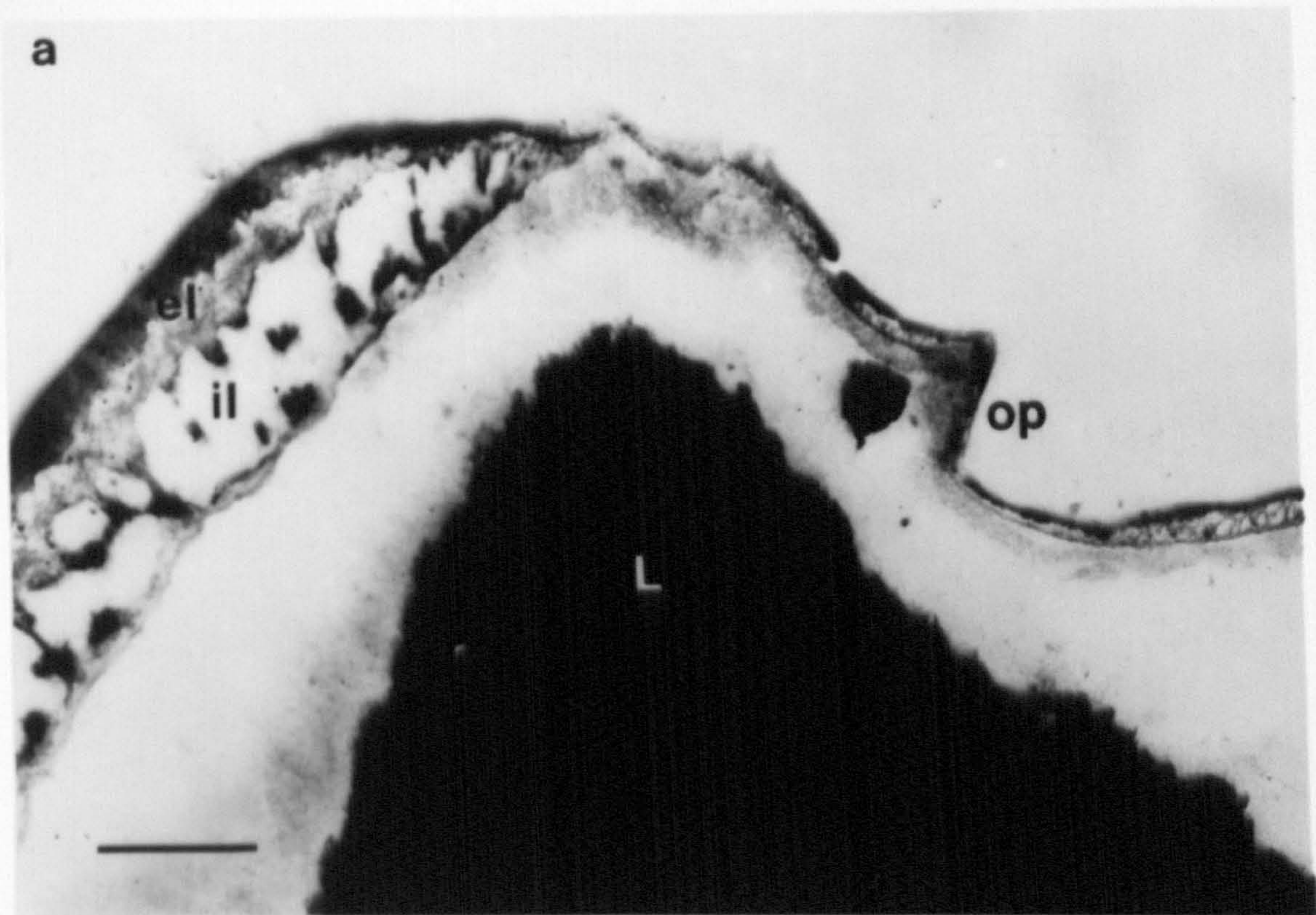


Plate 10

A. Transverse section through an unstimulated egg. Note the intact layers.
Mag.x17000. SB.=1 μ m.

B. Thin section through wall of unstimulated egg. Note the accumulation of particulate material (pm) in close proximity to the lipid layer (ll). Mag.x13500. SB.=1 μ m.

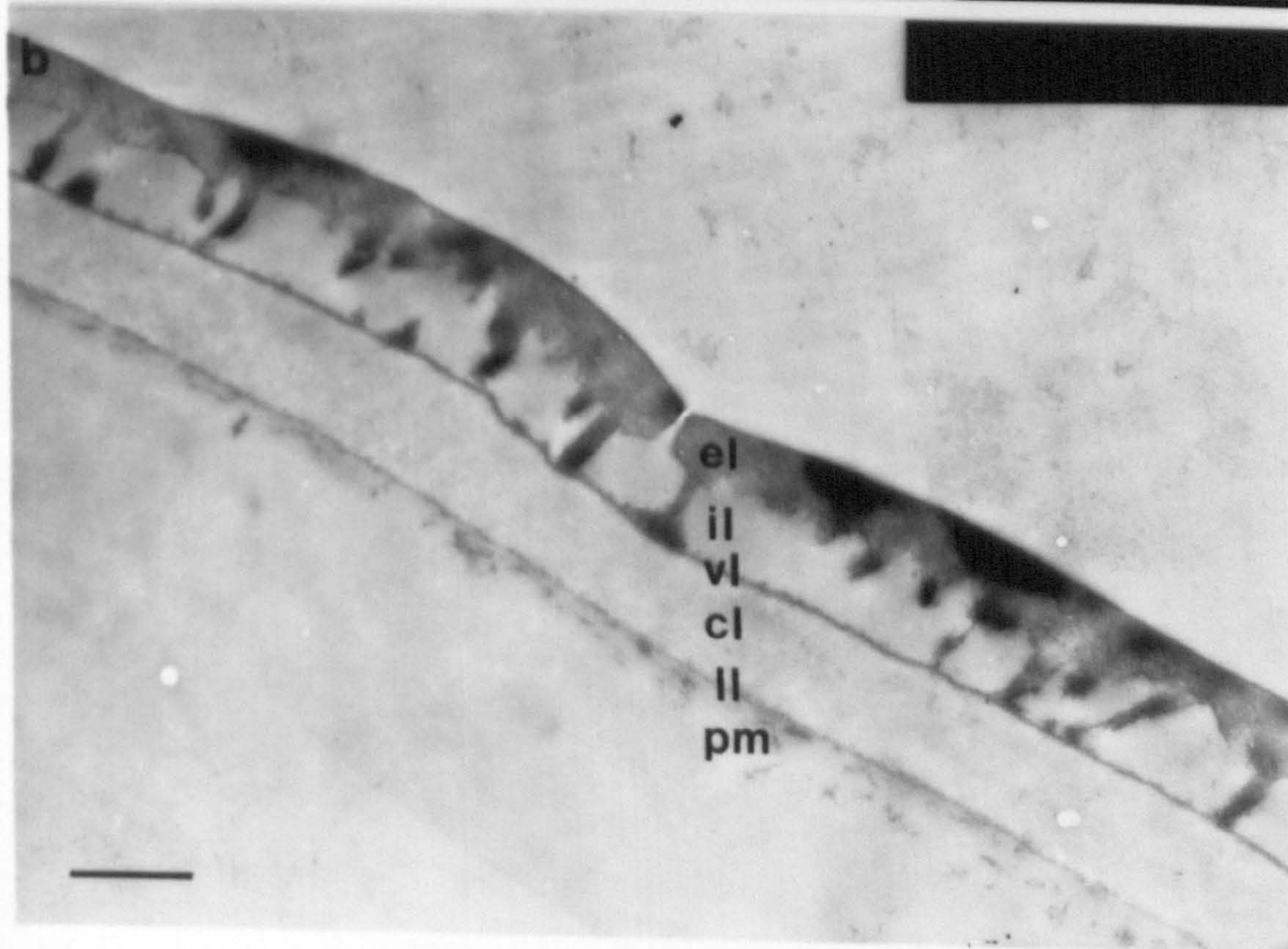
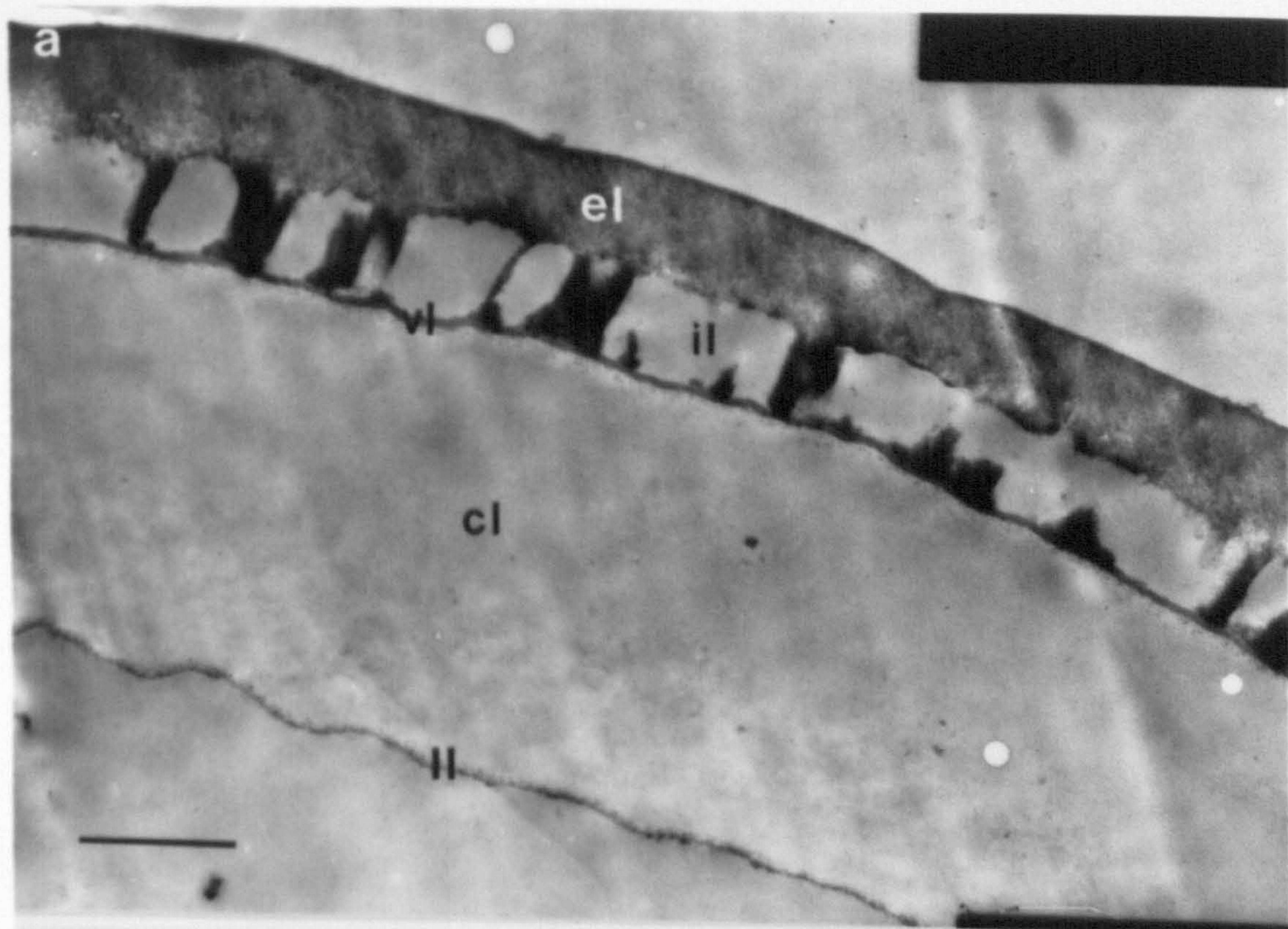


Plate 11

A.Intact operculum of egg, aged 5 days old, at 21°C, taken from a batch of eggs, prior to incubation at 37°C. Mag.x8500. SB.=2um.

B.Thin section through whole egg incubated for 2 hours in buffer at 37°C. The vitelline layer (vl) has begun to separate from the disintegrating internal uterine layer (il) and the chitin layer (cl) has deformed. Mag.x3000. SB.=6um.

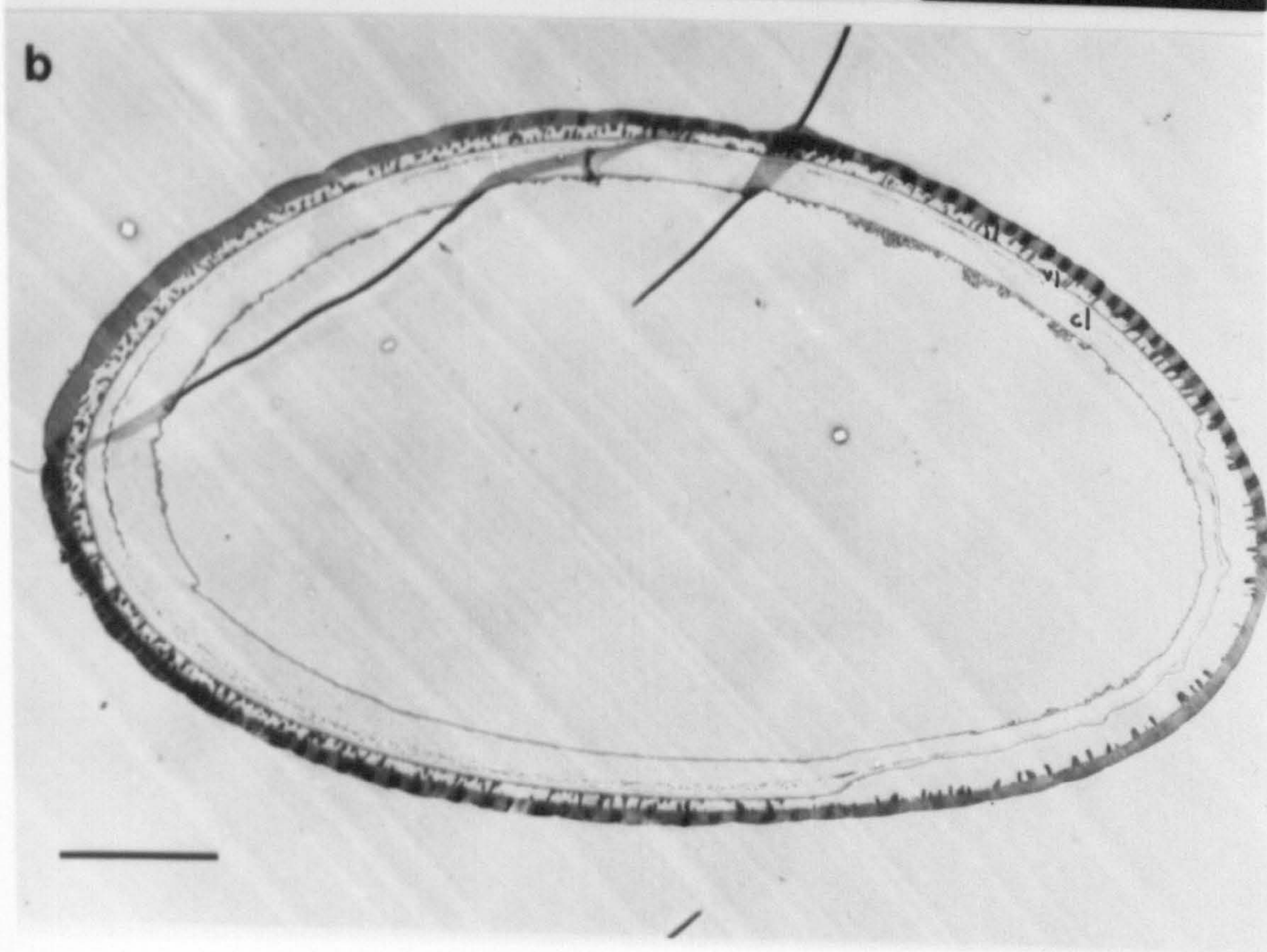
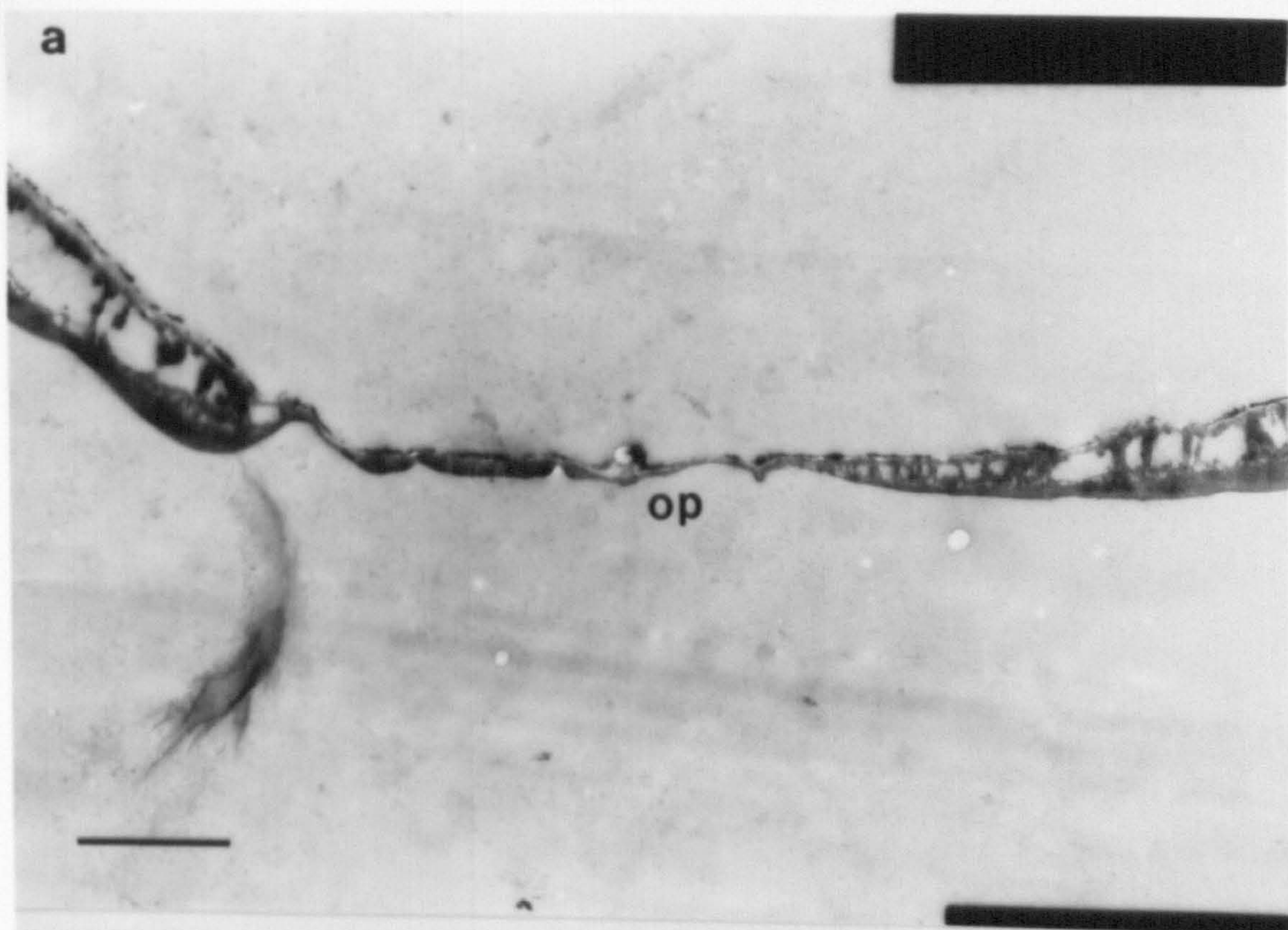


Plate 12

A.Thin section through egg incubated in buffer for 2 hours at 37°C. The struts of the internal uterine layer (il) have disintegrated and particulate material has accumulated alongside the lipid layer (ll). Mag.x13500. SB.=1um.

B.Increased magnification of eggwall from egg in Plate 12A, shows the separation of the vitelline layer (vl) from the struts of the internal uterine layer (il). Mag.x34000. SB.=500nm.

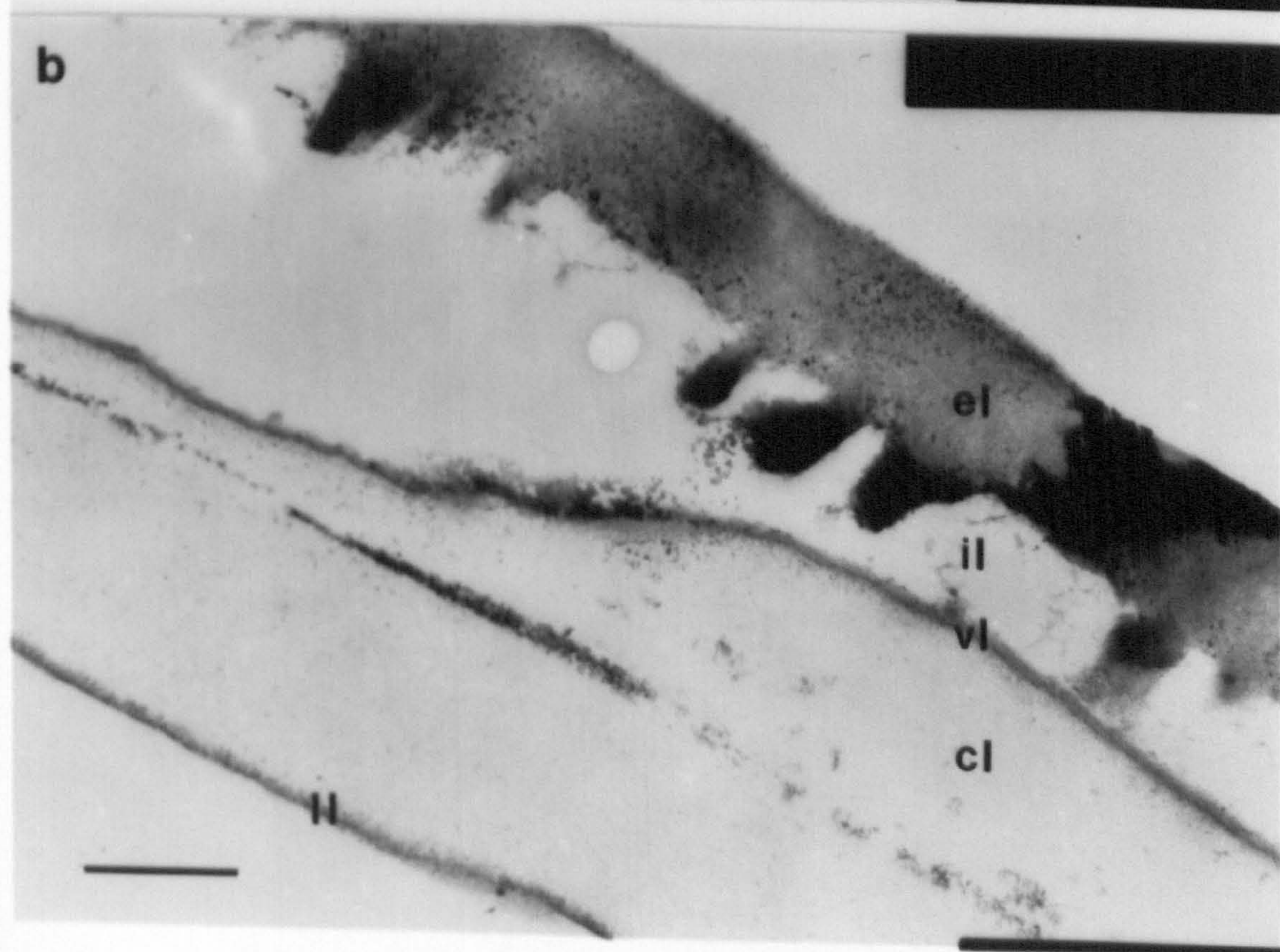
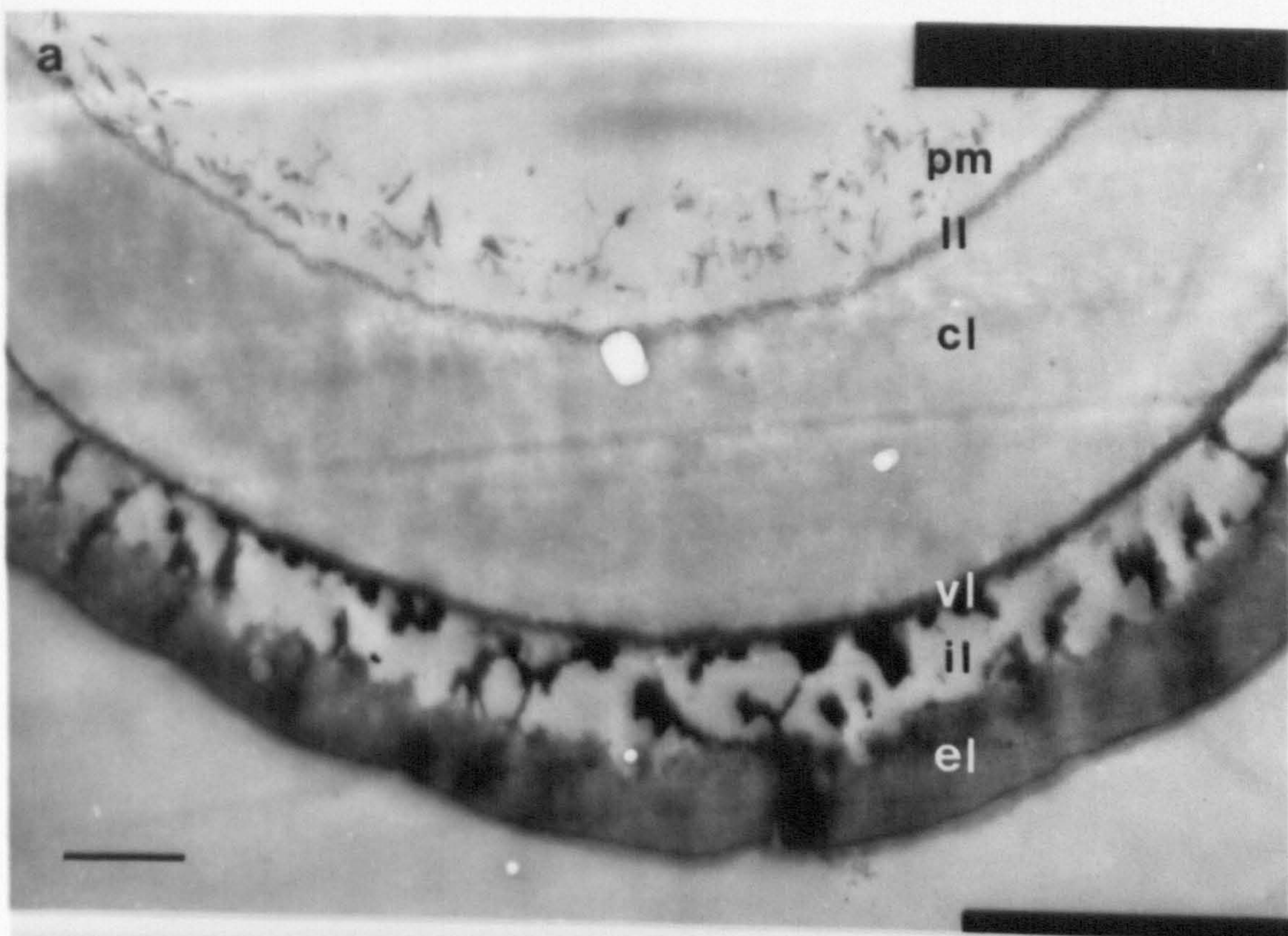


Plate 13

A. The underlying layers have separated from the uterine layers. The operculum (op) still remains intact. Mag.x6000. SB.=3um.

B. Eggshell of egg incubated in pepsin-HCl for 0.5 hour has softened, all the egg layers have deformed. The operculum (op) has remained intact. Mag.x3400. SB.=5um.

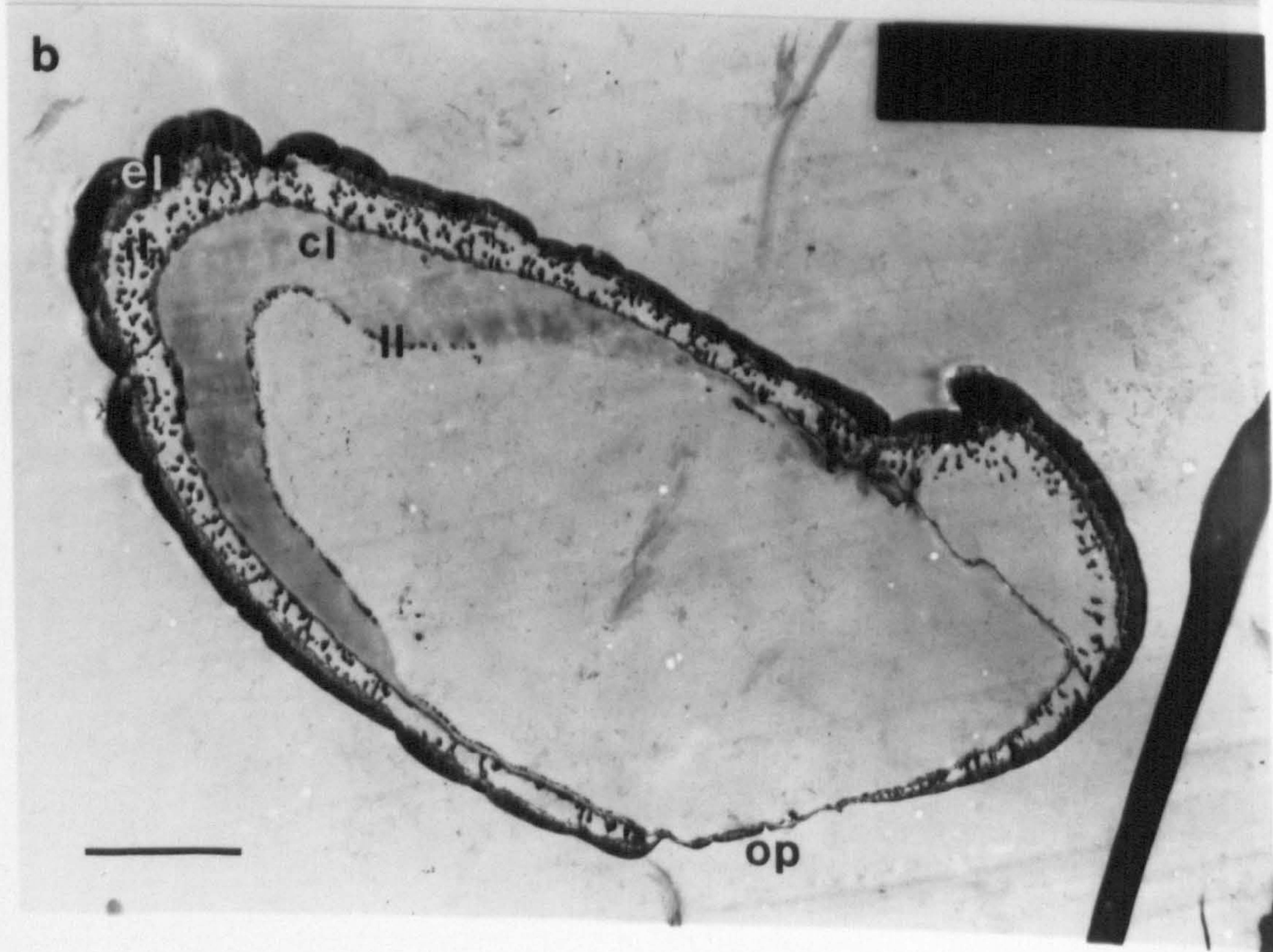
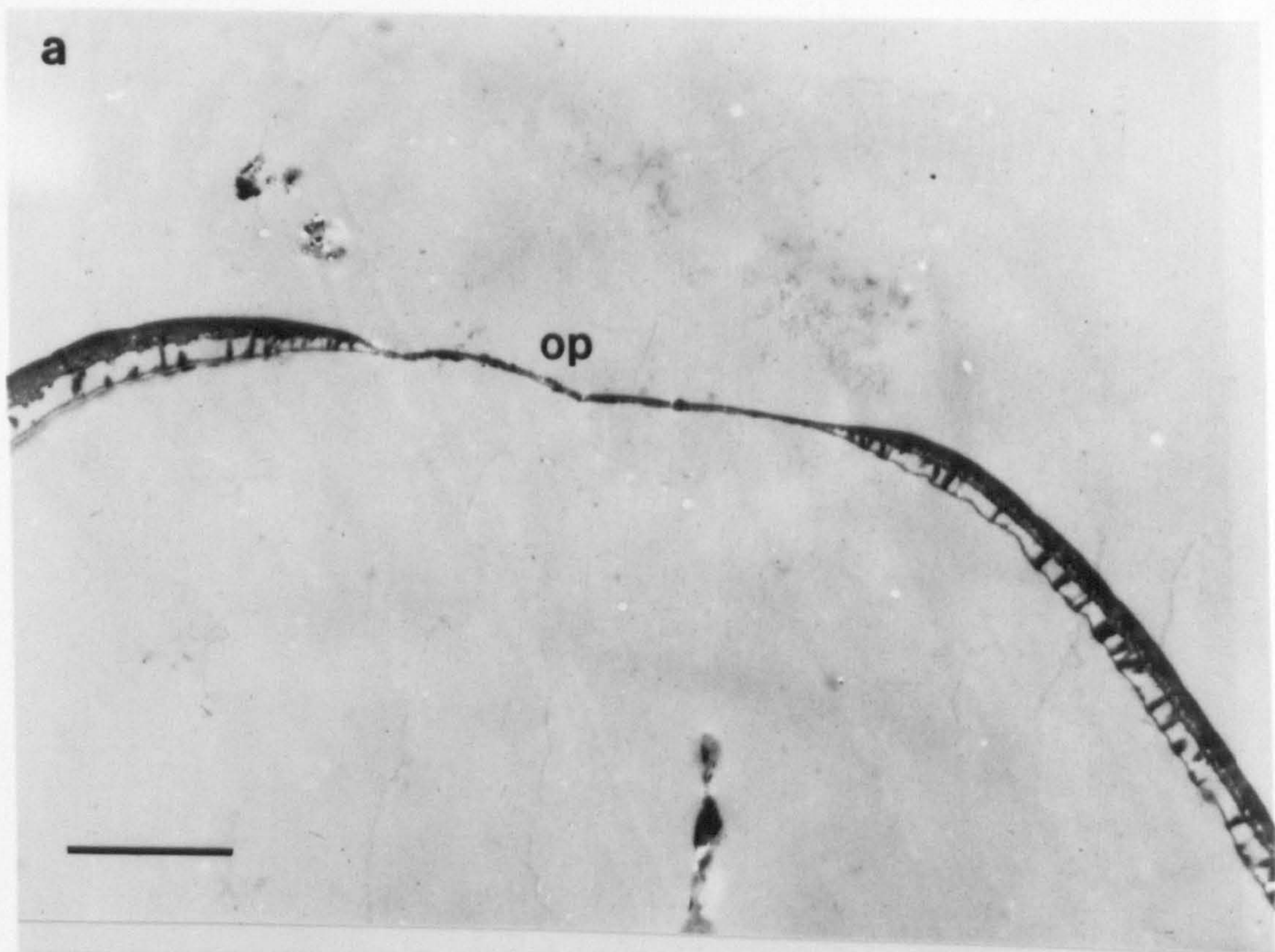


Plate 14

A. Egg incubated for 8 hours in buffer at 37°C. Note the increased disintegration of internal uterine layer (il), in comparison to the egg in Plate 12A. A space has formed where the chitin layer (cl) has separated from the underlying vitelline layer (vl). Increased accumulation of particulate material has occurred (pm). Mag.x5200. SB.=5um.

B Increased magnification of the operculum region of egg in Plate 14A. Note the operculum (op) has begun to disintegrate. Mag.x6800. SB.=2.5um.

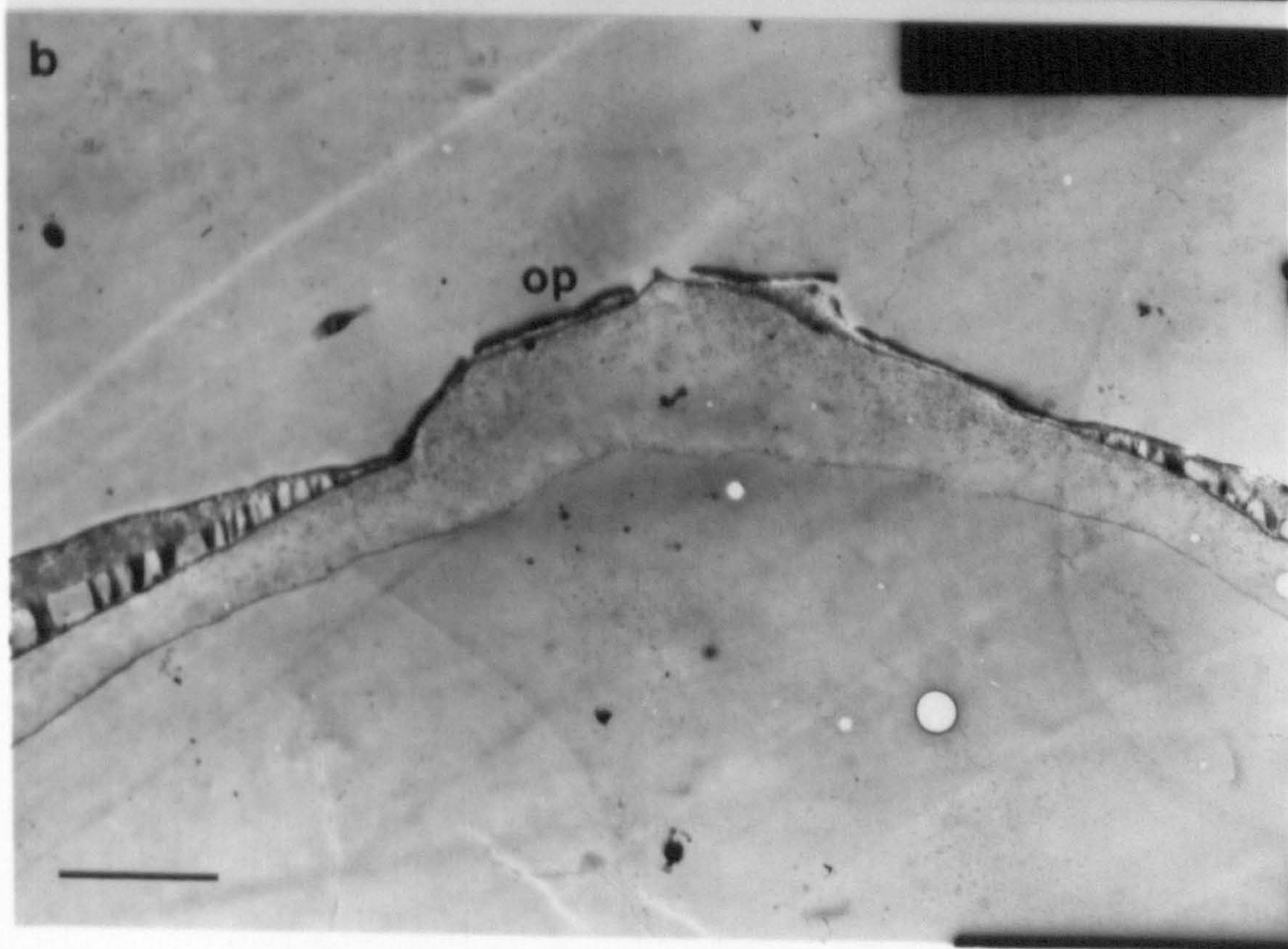
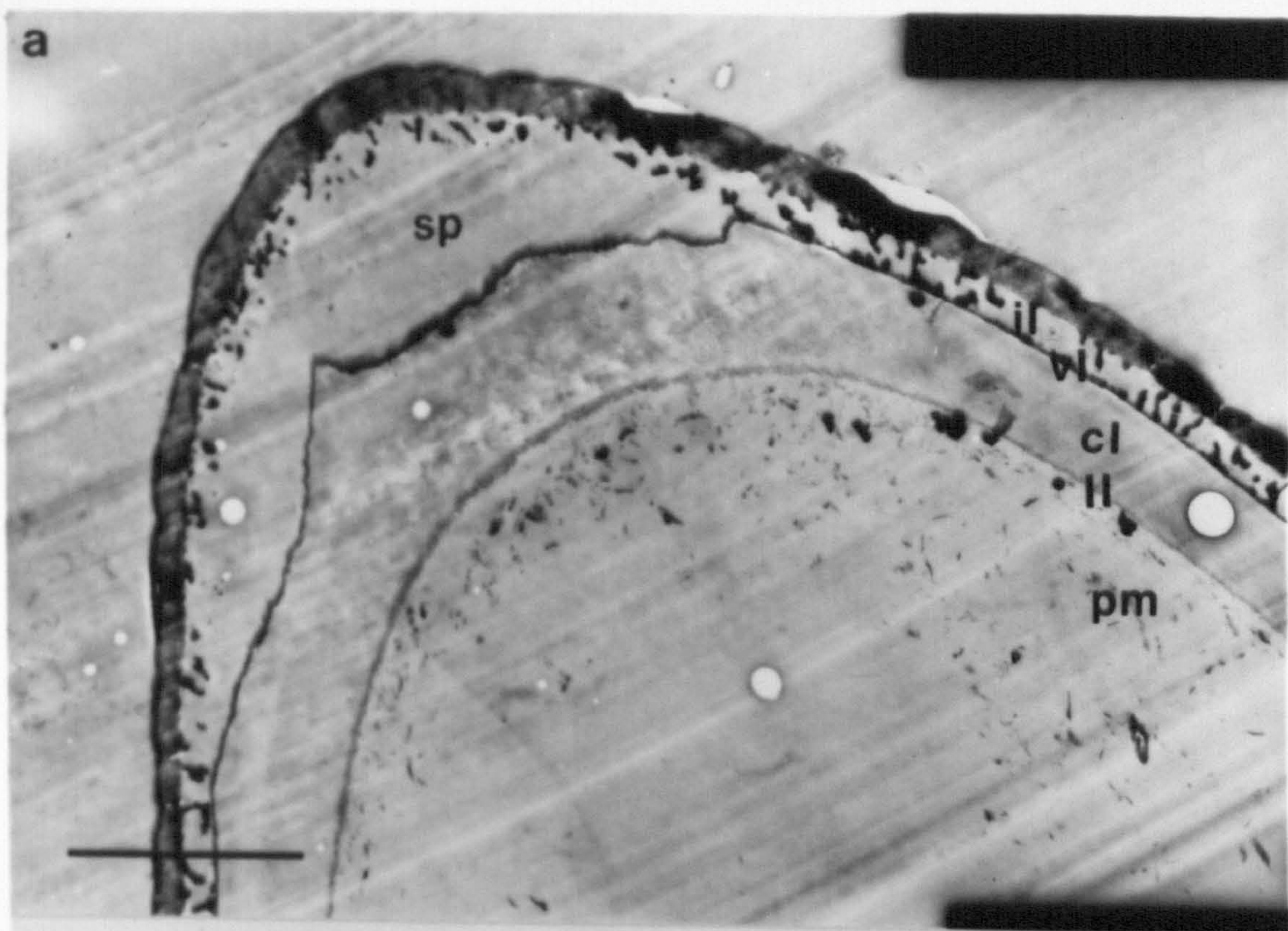


Plate 15

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A. Egg incubated in buffer for 8 hours at 37°C. The operculum (op) has opened and collapsed inwards trapping the larva (L) within the egg. Particulate material (pm) is present in close proximity to the lipid layer (ll). Mag.x3400. SB.=5um.

B. Increased magnification of egg in Plate 15A. A thin membrane (m) lies alongside the larva (L) which may be either a cast cuticle or the collapsed lipid layer. The particulate material (pm) may be the remains of the disintegrating lipid layer (ll). Mag.x8500. SB.=2um.

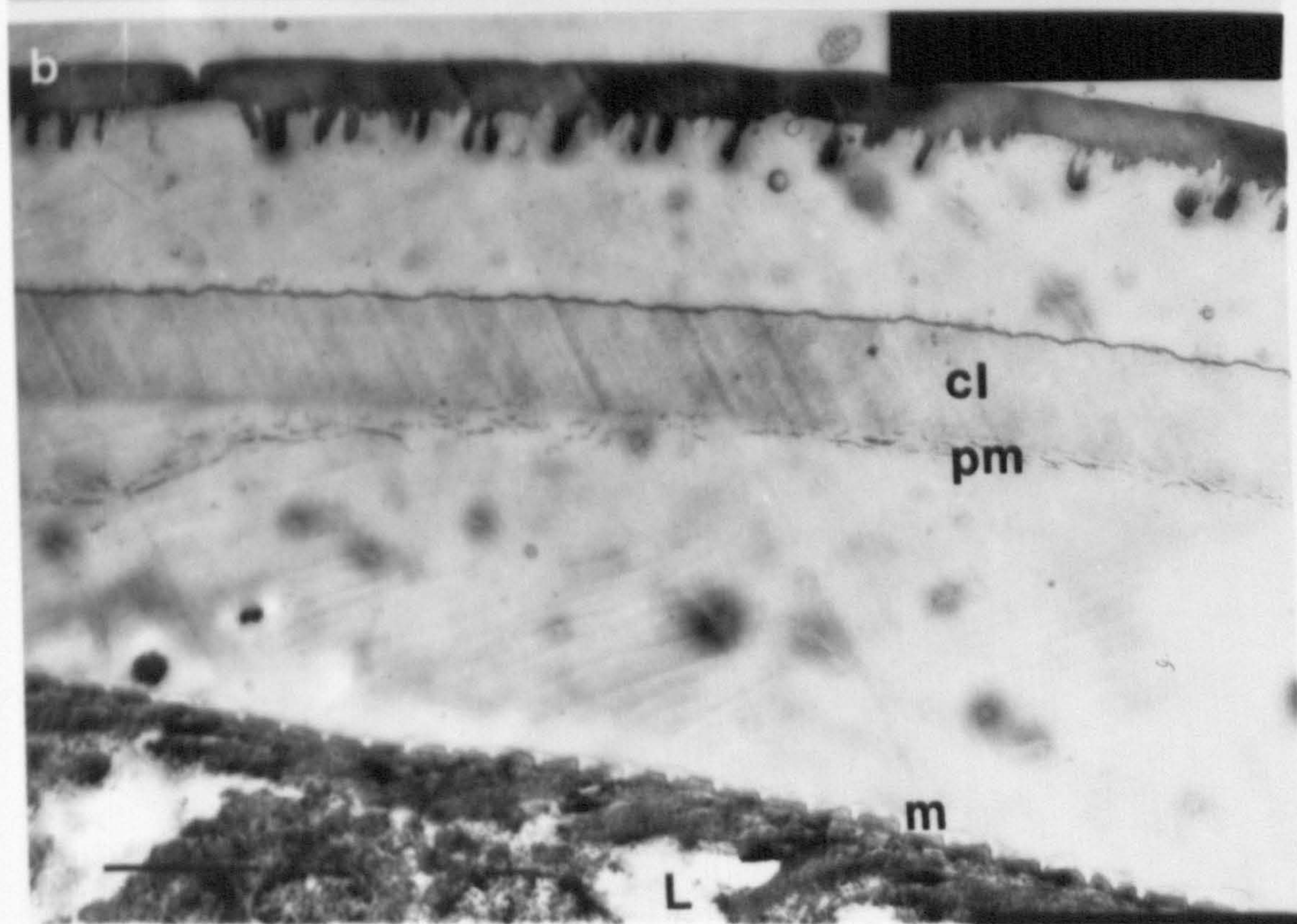


Plate 16

A. Egg incubated in dithiothreitol for 4 hours at 37°C. The chitin (cl) and lipid (ll) layer have collapsed around the larva (L). Mag. x10250. SB = 2µm.

B. Increased magnification of egg in Plate 16A. Note the separation of the lipid layer (ll), one half remains attached to the chitin layer (cl), the other half is attached to the larva (L). Mag. x17000. SB = 1µm.

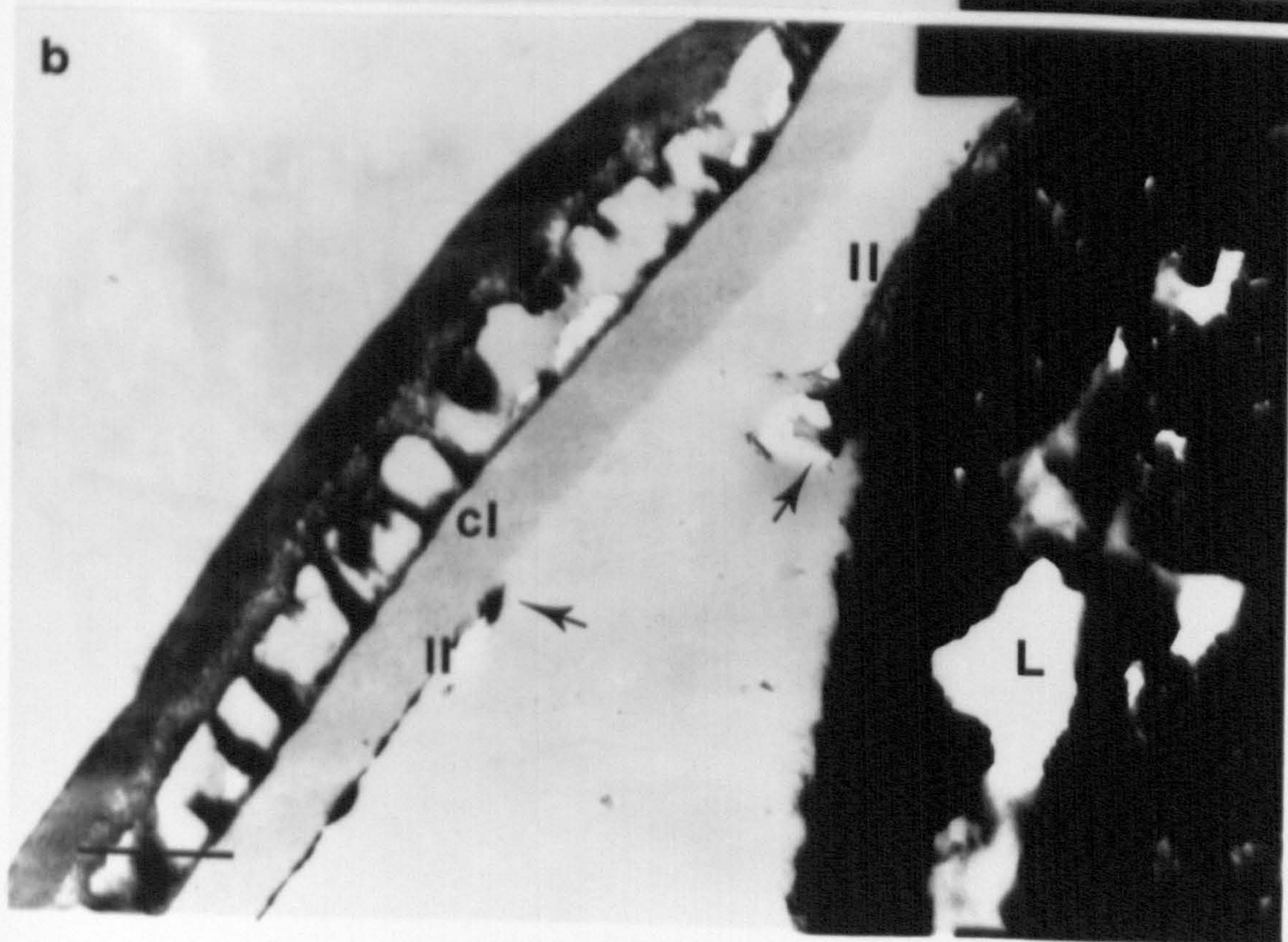
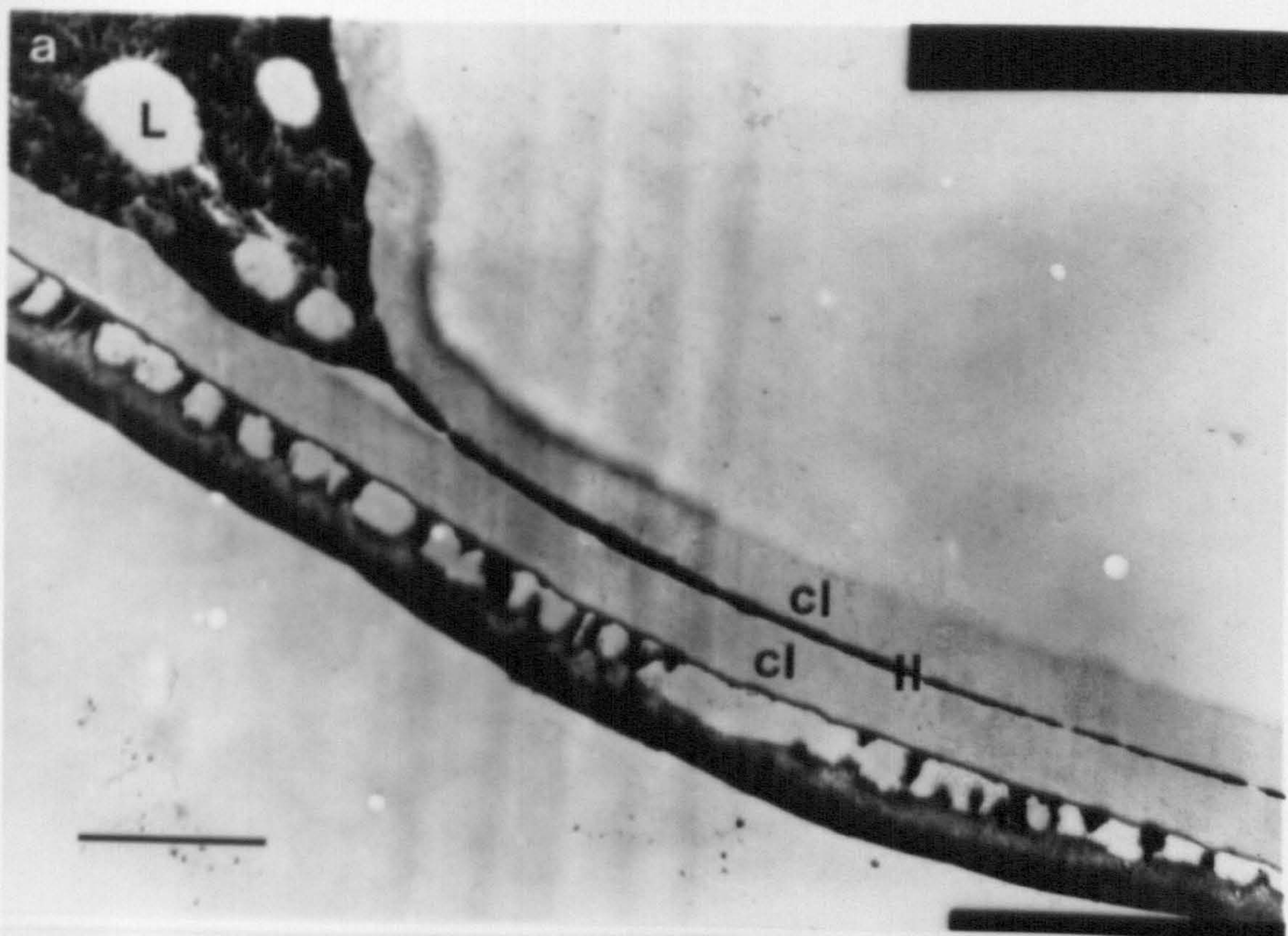


Plate 17

A Larva emerging through opercular region (op) of an egg incubated in pepsin-HCl for 2 hours at 37°C. Mag. x3400 SB=5um.

B Increased magnification of the larva from Plate 17A. Note the thin membrane (m) around the larva (L) and the disintegrated operculum (op). Mag. x17000. SB=1um.

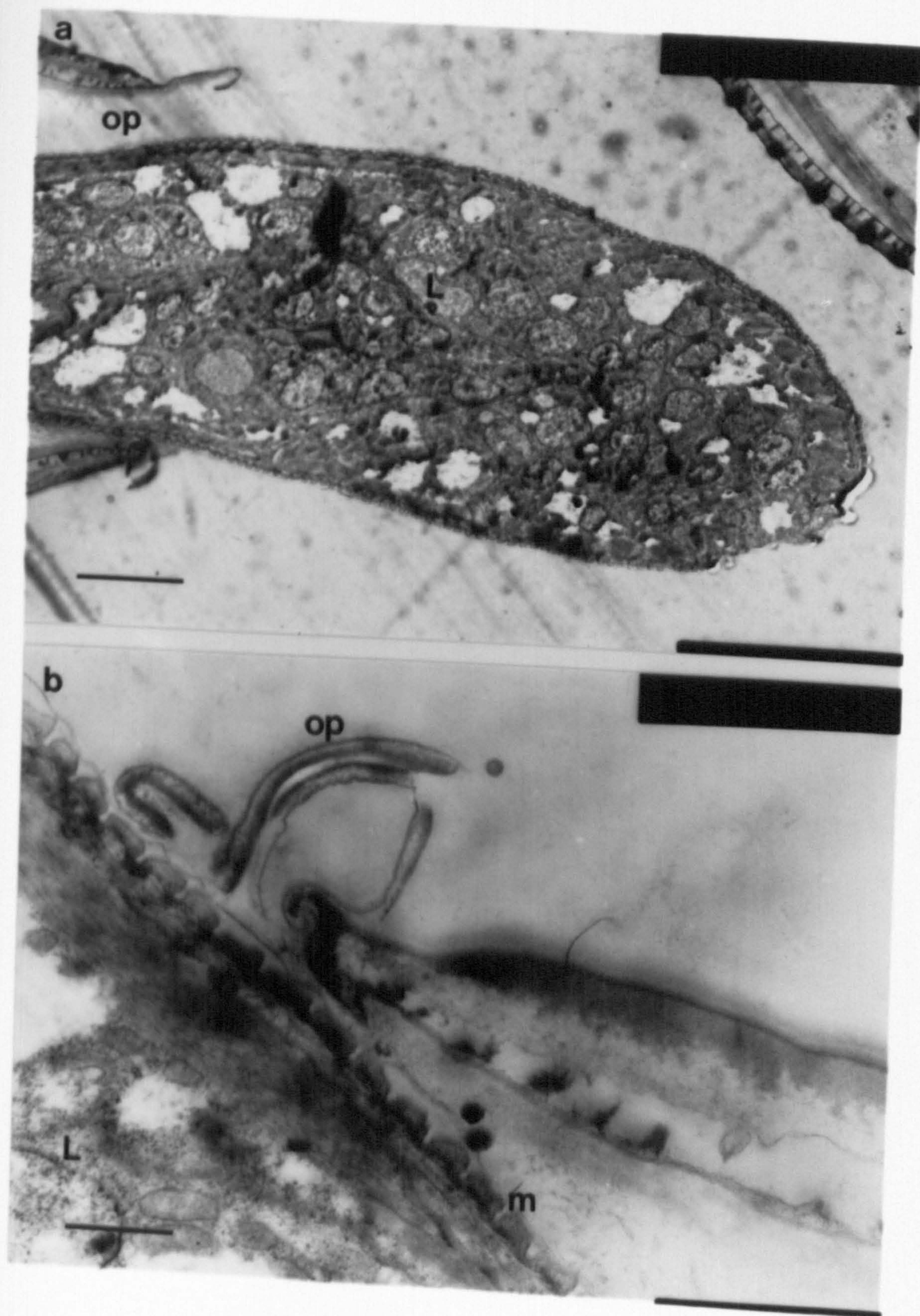


Plate 18

A. Empty egg incubated in buffer for 12 hours at 37°C. Note the open operculum (op) through which the larva has emerged. Mag.x5200. SB.=5um.

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B. Cross section through empty shell of an egg incubated in buffer at 37°C. Note collapsed vitelline (vl) and chitin layer (cl) and the disappearance of a portion of the lipid layer probably still attached to the larva (L). Mag.x4500. SB.=4um.

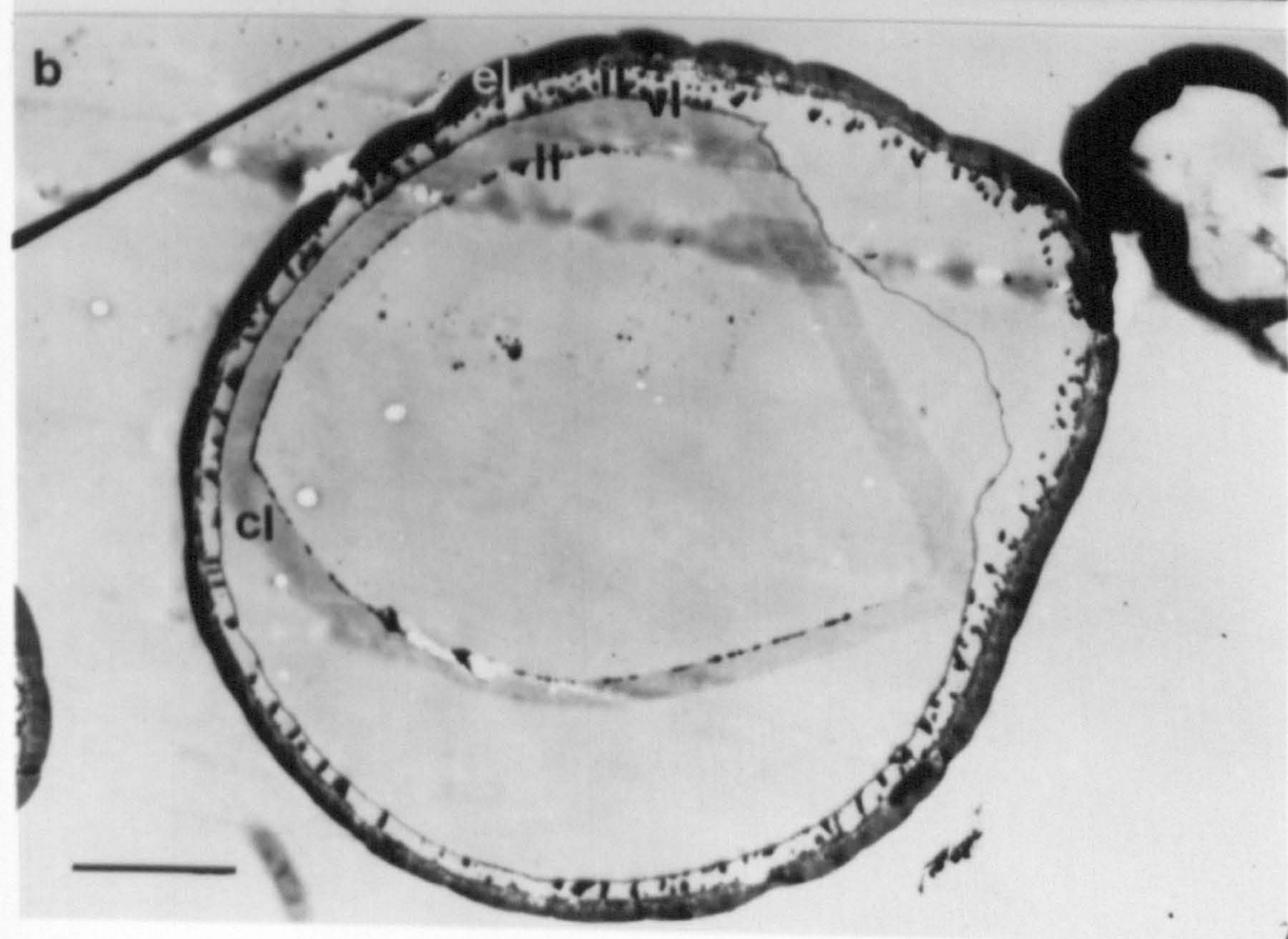
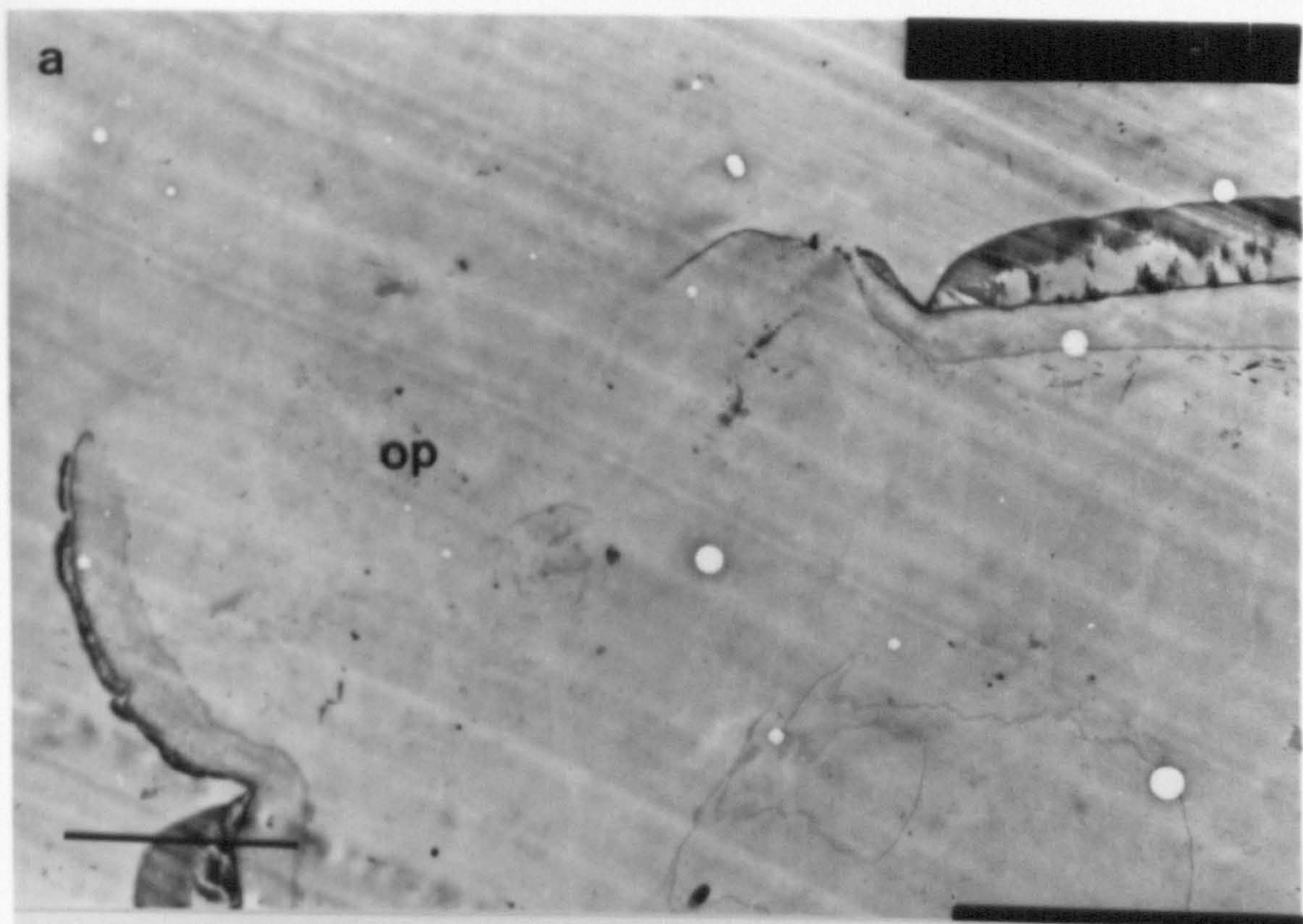


Plate 19

A.SEM of egg incubated in pepsin-HCl for 4 hours at 37°C prior to incubation in buffer at 37°C. Note the collapsed eggshell and emerging larva. The larva has pieces of membrane adhering to it's surface. Mag.x1000. SB.=20um.

B.SEM of egg incubated in buffer at 37°C for 8hours. Note the remnants of the operculum. The outer egglayer has retained it's rigidity and sculptured appearance at 37°C. Mag.x1575. SB.=20um.

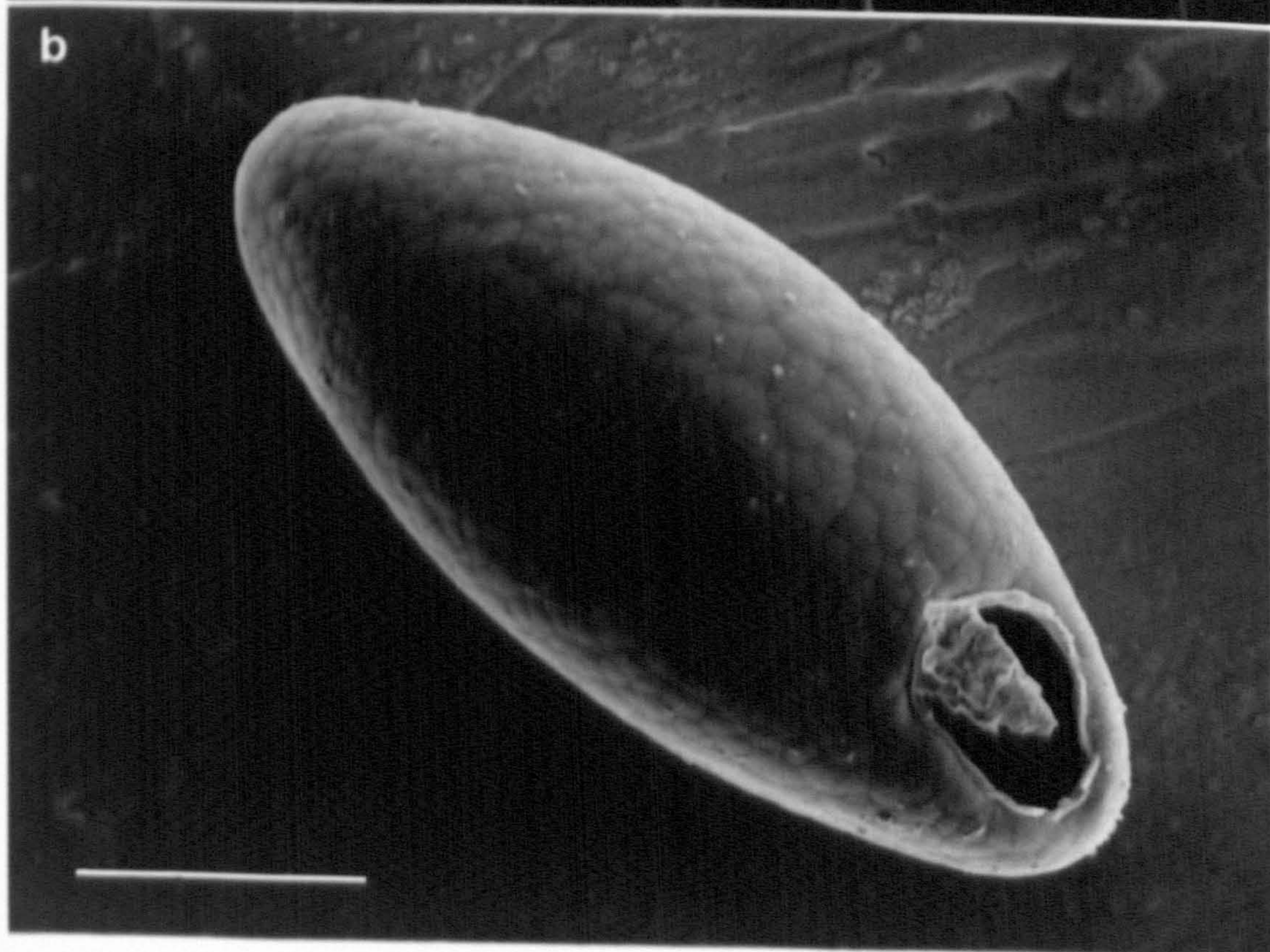


Plate 20

A.SEM of egg incubated in trypsin for 4 hours, prior to incubation in buffer at 37°C. Note the empty egg surface appears to be unaffected by the trypsin except in the opercular region. Mag.x1425. SB.=20um.

B.SEM of emerging larva from egg incubated in dithiothreitol for 4 hours at 37°C, prior to incubation in buffer at 37°C. Note adhering membrane on larva and collapsed eggshell. The unhatched egg lying in close vicinity to the hatching egg has collapsed but still retains an intact operculum. Mag.x600. SB.=20um.

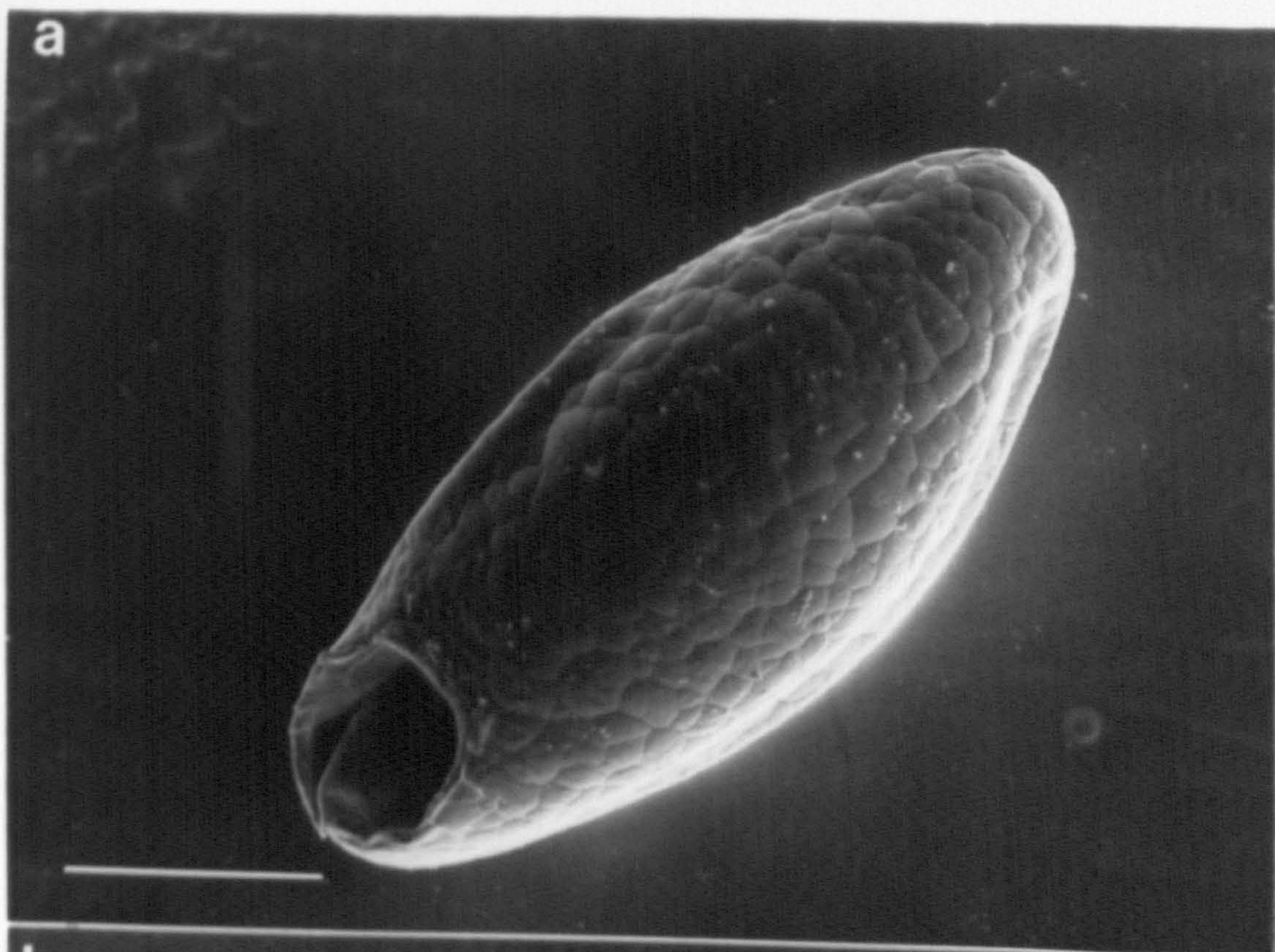


Plate 21

C. SEM of unstimulated egg at 21°C. Note similar appearance to egg surfaces of eggs incubated in pepsin-HCl, trypsin, dithiothreitol and buffer at 37°C (Plates 19 & 20). Mag.x1465. SB=20um.

D. SEM of unstimulated egg at 21°C. Note intact operculum. Mag.x3000. SB.=10um.

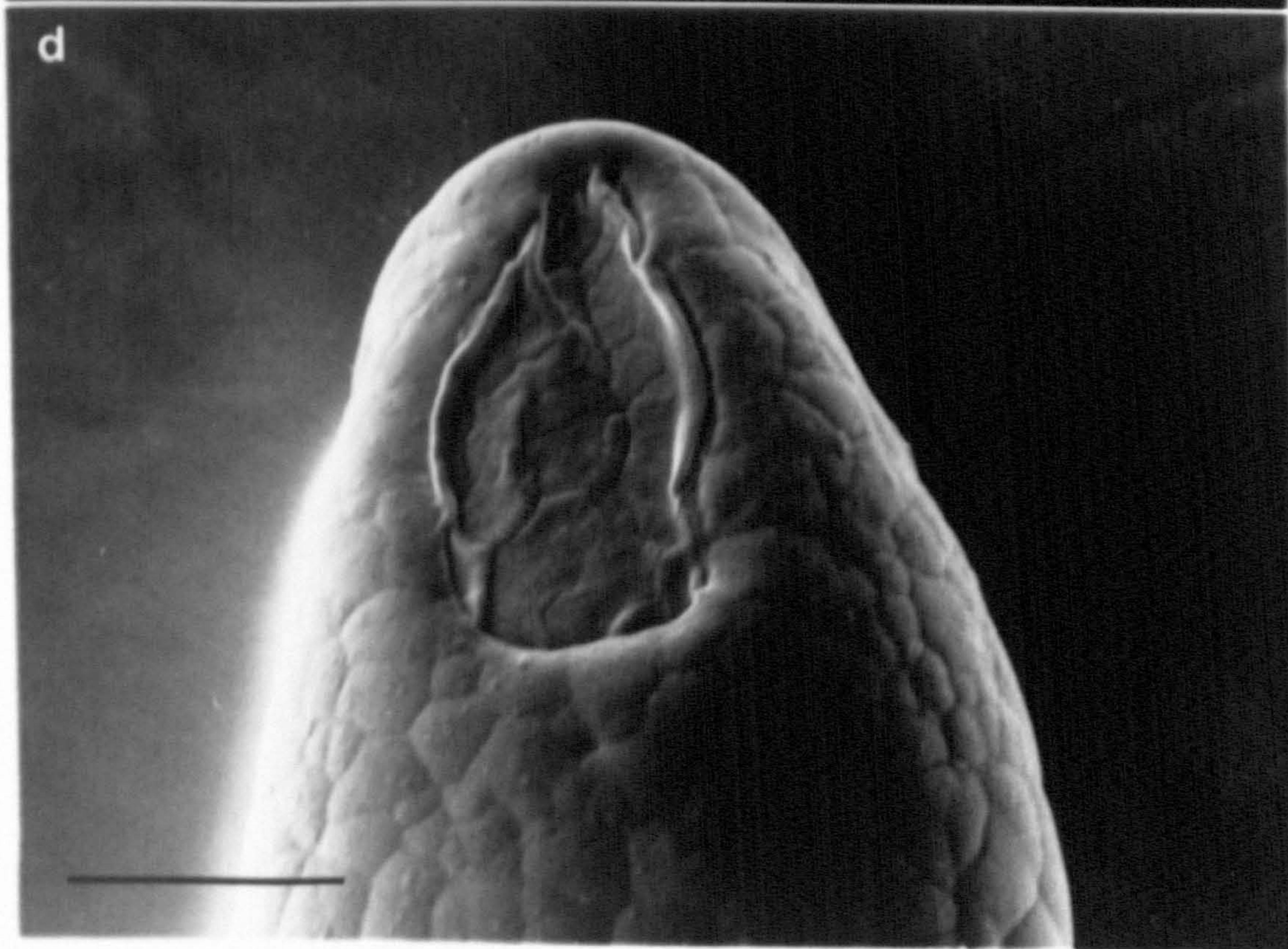
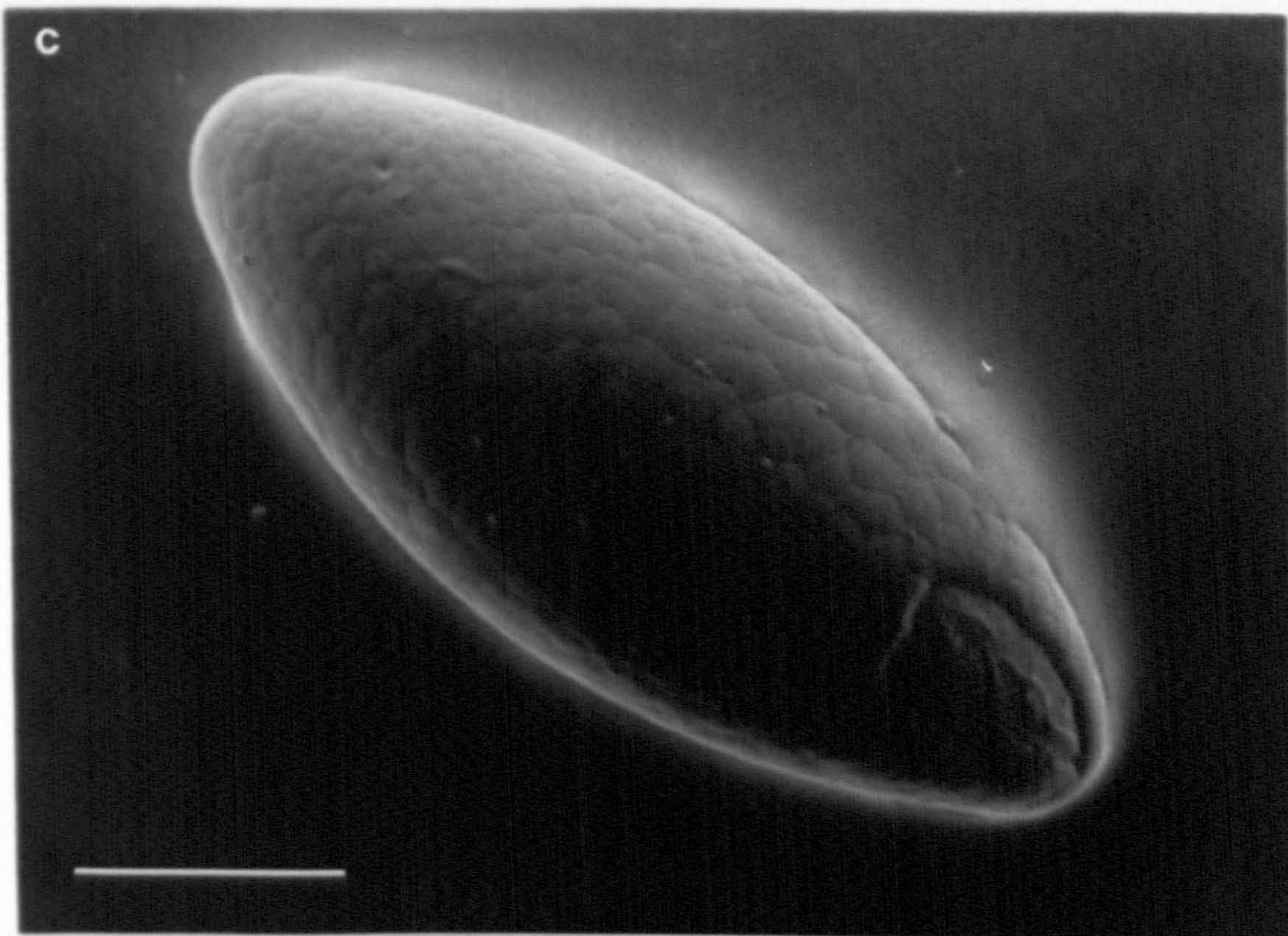


Plate 22

A. Egg stimulated to hatch by incubation in Bicine buffer (pH 8.5) at 37°C. Note enclosed larva (L) and collapsing egg membrane (cm). Mag.x1200. SB.=20um.

B. Larva emerging via the opened operculum of the egg in Plate 22A after 12 hours incubation at 37°C. Lipid layer (ll) and chitin (cl) have collapse around the larva (l). Note the rigidity of the outer eggshell has been retained. Mag.x1200. SB.=20um.

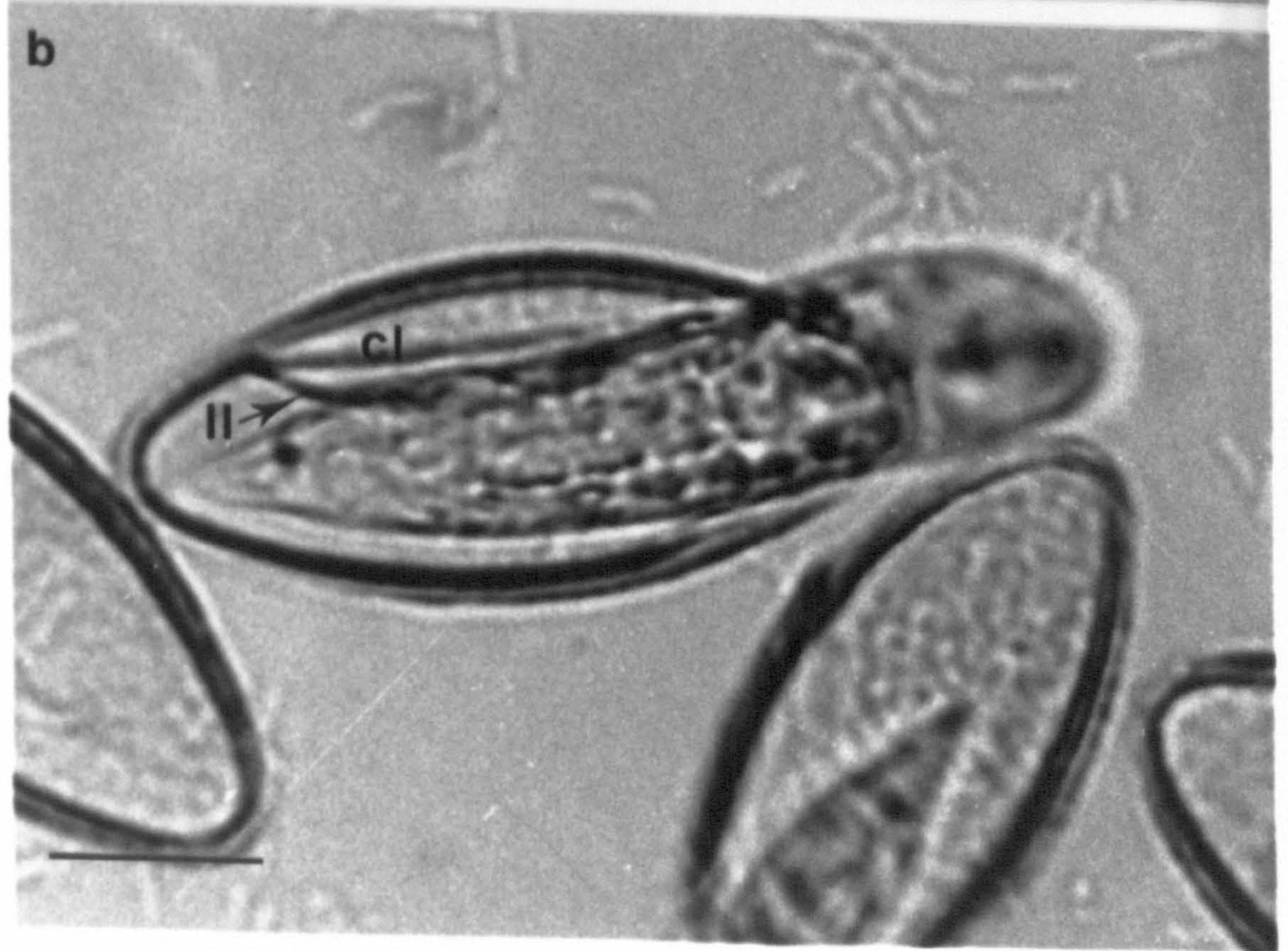


Plate 23

(L)
Larva_A fully emerged from the egg in Plate 22. Note the collapsed inner egg membranes (cm) and the opercular region (op) through which the larva emerged.

Mag.x1000. SB.=25um.

Abbreviations

cl:chitin layer; cm:collapsed membrane; el:external uterine layer; il:internal uterine layer; L:larva; ll:lipid layer; m:membrane; op:operculum; pm:particulate material; s:sheath; sp:space vl:vitelline layer.

Mag. = Magnification SB. = Scale Bar

